ROLE OF GRAVITATIONAL STRESS IN LAND PLANT EVOLUTION: THE GRAVITATIONAL FACTOR IN LIGNIFICATION

by
S. M. SIEGEL
and
C. H. LAMOUREUX, Co-investigator
BOTANY DEPARTMENT, UNIVERSITY OF HAWAII

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Submitted by: S. M. Siegel

S. M. Siegel
Professor of Botany &
Principal Investigator

National Aeronautics and Space Administration

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Plate I. Psilotum, one of two living genera in the Psilotales. This group belongs with the oldest known land vascular plants going back in fossil form to mid-Devonian times. Its lignin may represent the modern form of the most primitive type in existence, (approximate size, 70% of life size).

Plate II. Cucumber seedlings germinated under water with 10,000 ppm salinity. They are seen here with shoots emerging into the air, but note roots also doing so. ca. Twice life size.

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Introduction

This report presents the first phase of our pre-flight program under grant NGR 12-001-053. While the overall objective of the pre-flight period is obvious--the establishment of a workable basis for an eventual orbital experiment--the objectives of the present study also tend naturally to act as a collecting point for additional exploratory and supportive experiments for the basic hypothesis linking gravitational--mechanical stresses with lignification. These "background" studies have thus far fallen into two main categories; namely comparative biochemical and evolutionary or phylogenetic aspects of lignification and preliminary, general biological experiments under hyper-g conditions. The continued implementation of biochemical evolution is an essential for the intelligent formulation of new experiments and interpretations of current and future results in their broadest terms. The items of biochemical-phylogenetic import presented here involve a key enzyme in lignin polymer formation, peroxidase, which itself shows differential evolution among the several algal groups--evolution away from the ability to oxidize lignin precursor type structures in the non-green groups and retention of this capability in the green algae.

An important question pertaining to lignification has apparently been answered with the discovery of lignin in giant gametophyte stages of native New Zealand mosses. The absence of lignin from typical North American mosses only millimeters or a few centimeters in height plus their presence in substantial amounts in species 30 cm. in height or more suggests that lignification, although a gross taxonomic and phylogenetic feature of plants, may also be adaptive.

Hyper-g experiments are an essential phase of any gravitational study, but must be treated as an addition to hypo-g studies, and in no way as a replacement for hypo-g or zero-g effects. Growth and survival studies with insect larvae and seedlings are reported with responses of mangrove seedlings to centrifugal stress.

The second major section of this report is based upon an initial few pre-flight experiments. These experiments, and those now proceeding are intended to examine the behavior of the test plant selected--cucumber--under conditions relevant to hypo-g cultivation, to flight parameters, or both. Under relevant conditions, we include experiments involving orientation, clinostat technique, and buoyant media such as water. There are questions as to the similarities in clinostat and water culture methods in terms of plant response, and to the extent that they differ, which is a more valid ground-based approach to hypo-g or weightless conditions. These questions are not firmly answered yet. We hope that they will be in the next phase of the program to be reported in 1 May 1969. Pre-flight parameters include the substratum for seeds, containers, command module average or representative conditions of temperature, pressure, etc. Their evaluation will be superimposed upon the hypo-g experiments as they progress, but some are subject to separate study now.

Although it has been shown that clinostat and short-term orbital hypo-g conditions in Biosatellite II were at least qualitatively similar in wheat root and pepper plant assays, the ground-based history of the plants and short duration of orbital flight were

complicating factors. We hope to eliminate these problems and establish a workable procedure suitable for starting in-flight with dormant seed and allowing seedling growth to proceed for two full weeks, if possible, without attention until completion of the mission.

A-1 (a) Anomalous Substrate Patterns in Peroxidases From Red and Brown Algae

Peroxidase from horseradish, pea, and other vascular plants oxidizes guaiacylpropenes, e.g., eugenol and coniferaldehyde, to lignins in situ (Table A-1-1). In vitro these peroxidases readily oxidize guaiacyl derivatives, alkylphenols, monophenols, aromatic amines, pyrogallol and iodide. Peroxidases in green algae--e.g., Codium, Ulva, Nitella--resemble horseradish peroxidase, whereas 2 brown algae and 8 out of 9 reds tested failed to oxidize eugenol or guaiacol (Table A-1-2, Table A-1-3). The 2 browns (Postelsia [Fig. A-1-1], Sargassum) also could not peroxidize aniline or benzidine (Fig. A-1-2), typical horseradish peroxidase substrates. Peroxidase from all sources tested oxidized pyrogallol. Two reds failed to oxidize iodide, whereas 7 reds, 2 browns, 5 greens and horseradish peroxidase did so. Postelsia peroxidase did not oxidize IAA and was more sensitive to azide and cyanide (Table A-1-4). It appears that green algae having affinities with the ancestors of land forms possess a "lignifying" peroxidase whereas those specializing within the marine environment have "variant" peroxidases with more limited substrate ranges excluding lignin precursors.

Table A-1-1

A Summary of Peroxidase Substrate Test Conditions

DONOR SUBSTRATE	KI	PYROGALLOL	GUAIACOL	EUGENOL	ANILINE
Donor Conc. (mM)	10	10	10	10	10
H_2O_2 Conc. (mM)	2	20	20	20	20
Phosphate buffer (mM)	50	50	50	50	50
pH	8.9	4.5	6.1	6.1	6.1
Temperature (C)	24	24	24	24	24
Enzyme					
Horseradish (mg/ml)	0.1	0.1	0.1	0.1	0.1
Postelsia (mg dry matter/ml)	60	60	60	60	60
λ_{max} of Oxid Product (mμ)	420	425	470	425	600

Table A-1-2

A Summary of Substrate Specificity from Sixteen Algae

and Four Vascular (land) Sources

Species	Substrate				
	Pyrogallol 4.5	Guaiacol 6.1	Eugenol 6.1	Iodide 8.9	Aniline 6.1
Botryoglossum farlowianum	+	-	-	+	-
Catophyllis obtusifolia	+	-	-	-	-
Gelidium robustum	+	-	-	+	-
Gigartina californica	+	-	-	+	-
Gigartina corymbifea	+	-	-	+	-
Halymenia californica	+	-	-	+	-
Opuntiella californica	+	-	-	+	-
Porphyra sp.	+	-	-	+	-
Rhodymenia pacifica	+	-	-	-	-
Postelsia palmaeformis	+	-	-	+	-
Sargassum sp.	+	-	-	+	-
Codium edule	+	+	+	+	-
Ulva lactuca	+	+	+	+	-
Nitella sp.	+	+	+	+	-
Dictyosphaera sp.	+	+	-	+	-
Valonia sp.	+	+	+	+	-
Horseradish peroxidase	+	+	+	+	+
Elodea (cell free)	+	+	+	+	+
Pea root	+	+	+	+	+
Celery vasc. strands	+	+	+	+	+

Fig. A-1-1. Postelsia palmaeformis, are annual brown alga with erect form as shown here. Hollow thick-walled stipe is not woody--or lignified--but more rubber hose-like, and supports the plant body at low tide.

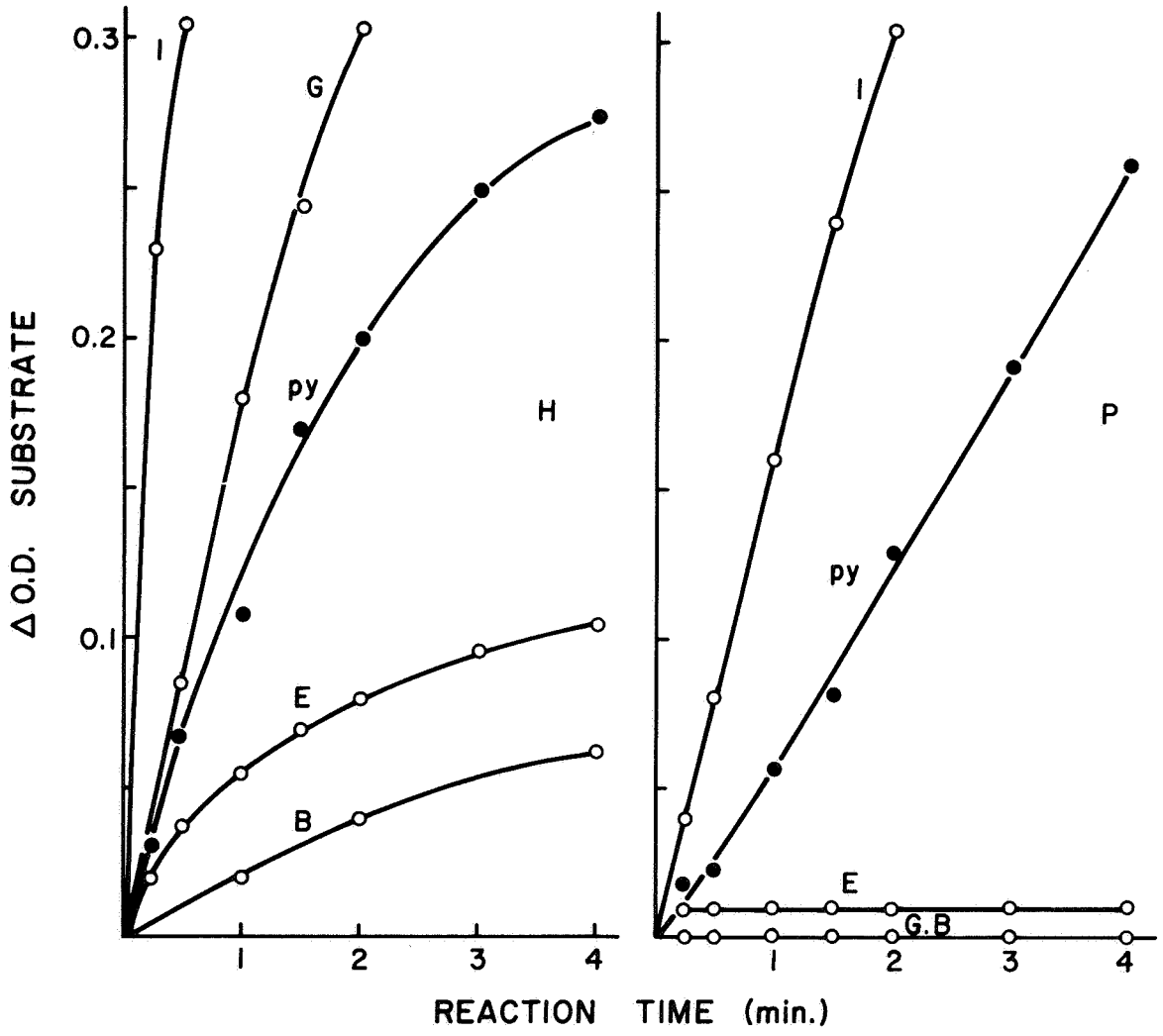

Fig. A-1-2. Peroxidase-substrate relations compared for typical horseradish enzyme and a preparation from the Brown alga, Postelsia. Substrate symbols: I = iodide, G = guaiacol, Py = pyrogallol, E = eugenol, B = benzidine.

Table A-1-3

A Summary of Substrate Activities of Horseradish Peroxidase

and Four Selected Algae

Substrate 10 mM (+20mM H_2O_2)	Horseradish	Postelsia	Sargassum	Ulva	Nitella
Iodide	+ (yell)	+ (yell)	+	+	+
Phenol	+ (pink)	-	-	-	-
Guaiacol	+ (red-brn)	-	-	±	±
3-Nonylphenol	-	-	-	-	-
Salicylic Acid	± (pink)	-	-	-	-
2,6-Dimethylphenol	+ (yell)	-	-	-	-
Resorcinol	+ (yell-or)	-	-	-	-
Pyrogallol	+ (or-brn)	+ (or-brn)	+	+	+
Eugenol	+ (turbid yell)	-	-	+	+
Isoeugenol	+ (turbid yell)	-	-	+	+
2-Naphthol	+ (yell-brn)	-	-	-	-
o-Phenylene Diamine	+ (purple)	+ (purple)	+	+	+
N-phenyl-p-phenylene diamine	+ (pur-brn)	-	-	-	-
Benzidine	+ (blue-grn)	-	-	-	-
2,7-Dihydroxynaphthalene	+ (pink→blue)	± (pink)	-	-	-
Aniline	+ (pink→black)	-	-	-	-

Table A-1-4

Some Comparative Properties of Purified Horseradish Peroxidase and Semipurified Postelsia Peroxidase

	Horseradish	Postelsia
Inhibitors	ID_{50} (mmoles/l)	
(Pyrogallol oxid.)		
NaN_3	9.2	0.8
KCN	4.8	1.5
Substrate Classes		
Monophenols	+	-
Guaicyl compounds	+	-
Polyphenols (ortho)	+	+
Aromatic amines	+	-
Indoles	+	-
Iodide	+	+
Heterogeneity		
(Iodide oxid.)		
Cationic Bands (pH 9)	3	0
Anionic Bands (pH 9)	2	1 (Smear)

A-1 (b) Lignification and Gigantism in Moss Gametophytes: Evidence for the Presence of Lignin in Dawsonia and Dendroligotrichum Species from New Zealand

A possible mechanical role for lignification in the upright land plant has long been recognized. It has also been shown in experimental studies on reaction wood, that lignification is subject to mechanical influences, and, from studies of xylem differentiation and regeneration, that it is also affected by non-mechanical factors such as auxin and oxygen partial pressure. More recently, a mechanical role for lignin has been confirmed by correlating increased compression force along the stem axis with regular basipital increases in lignin content.

Admittedly, lignification cannot be treated as the sole factor in erectness of the plant body. In particular cases hydrostatic pressure may support the aerial axis of aquatic plants, and infiltration of cellulose frames with silica provides an important alternative in Equisetum, many Graminae and other vascular forms. Nevertheless, lignification provides the most general means for increasing the bulk modulus of the cellulosic frame and increasing the dimensional stability of cells and tissues with respect to desiccation in the dry land environment.

Although the presence of lignin is a constant feature among vascular plants, including Psilotum, its distribution otherwise is extremely limited. It is reasonable, in fact, to generalize that among non-vascular plants, lignins do not occur in evident mechanical or supportive capacities and are virtually absent except in fruiting structures of wood-destroying Basidiomycetes.

Among typical North American bryophytes, lignin is absent from all stages in Marchantia, Riccia, Jungermannia, and the gametophytes of Bryum, Rhodobryum, Mnium, Polytrichum and Funaria. It was observed in the peristome teeth of the Polytrichum and Funaria capsules, however. From a bio-mechanical viewpoint, there is no basis for expecting lignification in "low-to-the ground" bryophytes. Even the pigmented, durable setae are devoid of any substance corresponding to lignin. Recently, however, the author had access for the first time to herbarium specimens of the gametophytes of two giant mosses native to New Zealand. The specimens of a Dawsonia sp. and a Dendroligotrichum sp. ranged from 35-55 cm in length, an order of magnitude taller in general than the moss gametophytes examined previously. The size of these slender gametophytes was well within the range of vascular plants, including semi-woody and woody forms, hence making them subject to similar mechanical stresses. Although bryophytes have been characterized as neither containing nor requiring lignin in a supporting capacity, the existence of lignin in some peristome teeth and in other non-vascular groups (e.g. basidiomycetes), no matter how specialized left open an important question: Does the distribution of lignin adhere more or less rigorously to systematic-phylogenetic lines, or alternatively, as an adaptive character, can it be found in groups in which it is otherwise characteristically absent?

The presence of lignins in substantial amounts in the axes of giant gametophytes would immediately support further their assumed mechanical role and broaden their evolutionary significance.

Gametophytes of Dawsonia and Dendroligotrichum were provided from the collection of Prof. C. Lamoureux, Department of Botany, University of Hawaii. Analyses of Polytrichum gametophytes collected in New York state were carried out in 1958-60, and Psilotum was collected in the Manoa district of Honolulu.

Preliminary phloroglucinol tests for lignin were positive in gametophyte axes, but negative in the leaves of the large mosses. Accordingly, axes were stripped free of appendages before solvent and chemical treatment. Routinely, pulverized axes were extracted with cold 1M $NaHCO_3$, hot 50% ethanol and hot chloroform (100 ml/g dry tissue) to remove most organic acids, sugars, amino acids, phenols (including lignin precursors), lipids and other solubles that might interfere with lignin tests and analyses. These fractions gave negative phloroglucinol tests for lignin.

Ethanolysis was carried out with boiling 95% ethanol-3% HCl. This procedure solubilizes lignin by a combination of solvolysis and depolymerization. Solids were obtained after ethanolysis by concentration at 50 degrees C/25 mm Hg pressure followed by drying at 65 degrees C/0.1 mm Hg. Isolation of Klason lignin involves cold digestion with 72% H_2SO_4 followed by hydrolysis with dilute (e.g. 3%), acid, neutralization and washing. Cold strong sulfuric acid swells and partially degrades celluloses, rendering them hydrolyzable in hot dilute acid; lignin forms the insoluble residue. Cuprammonium solution (Schweizer's reagent) also removes cellulose leaving lignin largely undissolved. The isolated material is sometimes termed "cuoxam" lignin. Sodium hydroxide solution, solubilizes weakly acidic substances such as phenols, including lignins.

Standard color tests for lignins include phloroglucinol in HCl, Cross and Bevan chlorine-sodium sulfite treatment, and Maüle test. Lignins give a characteristic red-violet color with phloroglucinol-HCl; chlorination followed by sulfite treatment after Cross and Bevan yields a red coloration with angiosperm lignin and a yellow color with lignin from other sources. The Maüle test--also based upon chlorination-distinguishes gymnosperm and angiosperm lignins by characteristic brown and red color reactions respectively. The syringyl, or pyrogallol dimethyl ether group of angiosperm lignin reportedly accounts for the color difference. Other standard chemical tests include specific reagents for aromatic nuclei, phenols, reducing agents and carbonyl compounds.

Isolated samples were subjected to nitrobenzene oxidation, releasing compounds characteristic of the monomeric units comprising lignin: p-hydroxybenzaldehyde, vanillin and syringaldelyde. The specific alkaline nitrobenzene procedure used here was the micromethod of Stone and Blundell, however the resultant aldehydes were precipitated collectively as their 2,4-dinitrophenylhydrazones, using 10% solution of 2,4-dinitrophenylhydrazine in 35% ethanol.

Elementary and group analysis for C, H and OCH_3 were carried out by commercial analytical services.

Frequently direct tissue tests with color reagents are more sensitive than the same tests carried out on extracts. Furthermore, tissue provides a vehicle for the successive operations of the Cross and Bevan and Maüle tests (Table 1). The tissue after treatment 1 is used for color standards, but actually differs little in response to color tests from unextracted material. Dawsonia and Dendroligotrichum gametophytes yield

Table 1

Lignin Color Reactions of Gametophyte Tissue and

their Modification by Solvent Treatment

Treatment		Species			
Solvent	Color Test[1]	Dawsonia	Dendroligotrichum	Polytrichum	Psilotum[2]
1. Bicarbonate-50% Ethanol-Chloroform	Phloroglucinol	RV	RV	yl	RV
	Cross and Bevan	Or	Or	-	Or
	Mäule	Br	Br	-	Br
2. Ethanol-HCl ("Ethanolysis")	Phloroglucinol	RV-	RV-	yl	RV-
	Cross and Bevan	Or-	Or-	-	Or-
	Mäule	Br	Br	-	Br
3. 72% Sulfuric Acid ("Klason")	Phloroglucinol	RV-	RV-	-	RV-
	Cross and Bevan	Or	Or	-	Or
	Mäule	Br	Br	-	Br
4. Ethanol-HCl on 3 Residue	Phloroglucinol	-	-	-	-
	Cross and Bevan	Or-	Or-	-	Or-
	Mäule	Br-	Br-	-	Br-
5. Cuprammonium	Phloroglucinol	RV+	RV+	-	RV+
	Cross and Bevan	Or	Or	-	Or
	Mäule	Br	Br	-	Br
6. 1M NaOH	Phloroglucinol	tr	tr	-	tr
	Cross and Bevan	tr	tr	-	tr
	Mäule	tr	tr	-	tr

[1] RV=red violet, Or=orange, Br=brown; RV+ = intensified, RV- = weaker, etc., tr = trace

[2] Sporophyte

typical phloroglucinol tests for lignin as does the sporophyte of Psilotum, whereas the small gametophyte of Polytrichum gave only a yellow (negative) color. The typical Mäule test of non-angiosperm was given by all samples but Polytrichum, and the Cross and Bevan test yielded an orange reaction (as opposed to red in angiosperms). Polytrichum was negative again.

Ethanolysis reduced the intensities of phloroglucinol and Cross and Bevan tests, but effected no subjective change in the Mäule brown reaction. The Klason (72% sulfuric acid) residue also exhibited an attenuated phloroglucinol reaction, a result of partial destruction of functional groups, but the Cross and Bevan and Mäule colors were unchanged. Ethanolysis of the Klason residue leaves a material devoid of phloroglucinol chromogen and reduced in other color responses. Cuprammonium treatment did not remove all the cellulose, but enhanced the phloroglucinol reaction in all samples except Polytrichum but no enhancement was noted in the other color test. After alkali extraction virtually all chromogens were removed from the tissue.

Phloroglucinol reactions in the tissues were complemented by tests upon the more important extracts (Table 2). They show the strong color of direct ethanolyses, and attenuation of fractions from Klason residues. Alkali removed nearly all chromogen from the tissues, but it did not yield a strong color, a consequence, possibly of alkaline autoxidation.

The yellow brown solid concentrate from ethanolysis nitrates to deep yellow products turning intense orange with excess ammonia water, typical behavior of aromatic nuclei, especially phenols (Table 3).

Table 2

Phloroglucinol Reactions in Gametophyte Extracts

Solvent	Phloroglucinol Color of Extract[1]			
	Dawsonia	Dendroligotrichum	Polytrichum	Psilotum[2]
50% Ethanol-Chloroform	-	-	-	-
Ethanol-HCl	RV	RV	yl	RV
Ethanol-HCl on Sulfuric Acid Residue	RV-	RV-	-	tr
1M NaOH	RV-[3]	RV-	yl	RV-

[1] See footnote, Table 1

[2] Sporophyte used

[3] Color reagent applied to NaOH extracts after neutralization with HCl

Table 3

Color Reactions and Chemical Tests with the Ethanol-HCl Fractions from *Dawsonia* and *Dendroligotrichum*

Reagent	Source of Preparation	Reaction	Interpretations
Conc. HNO_3	*Dawsonia*	deep yellow	aromatic nucleus
	Dendroligotrichum	deep yellow	aromatic nucleus
NH_4OH to above	*Dawsonia*	deep orange	confirmatory to above
	Dendroligotrichum	deep orange	confirmatory to above
0.05 M $FeCl_3$ in 95% Ethanol	*Dawsonia*	pale blue green	polyphenol
	Dendroligotrichum	pale blue green	polyphenol
Folin Phenol Reagent	*Dawsonia*	pale blue	phenol
	Dendroligotrichum	pale blue	phenol
Fehlings Solution	*Dawsonia*	red-orange ppt.	reducing groups
	Dendroligotrichum	red-orange ppt.	reducing groups
2,4-Dinitrophenylhydrazine	*Dawsonia*	red ppt.	Hibbert Ketones or aldehydes
	Dendroligotrichum	red ppt.	Hibbert Ketones or aldehydes

Phenolic character was confirmed using $FeCl_3$ and Folin reagent. Reducing character was also evident, as was the presence of 2,4-dinitrophenylhydrazine-precipitable "Hibbert ketones". Ethanolytic residue from both gametophytes gave qualitatively identical results. Once isolated, the ethanolysis product was found to be soluble in hot methanol, ethanol, dioxane, NaOH solution and slightly soluble in ammonia, but insoluble in water, ethers and hydrocarbons. The Klason isolate was not soluble in neutral solvents, but dissolved well in alcohol-acid and alcoholic alkali.

The ultraviolet absorption maxima at λ280-282 mu found in ethanol-HCl extracts from tissues and Klason residues (Table 4) are completely typical of most lignins. Alkali extracts show the characteristic spectral shift to longer wavelength. Psilotum extracts were similar and Polytrichum extracts yielded only weak end-absorption in the ultraviolet.

The phloroglucinol-HCl spectra obtained upon ethanolysis of gametophyte tissue show usual maxima at λ540-542 mu together with a second maximum at a shorter wavelength. Psilotum shows only the usual maximum near λ540 mu, as do the alkali extracts in all cases.

Lignins isolated from Dawsonia and Dendroligotrichum by the Klason method or by ethanolysis gave substantially similar results (Table 5). Lignin content ranged from 6.1-10.4% consisting of ca. 61-62% C, 6.4-6.8% H and 5.1-7.9% OCH_3. The carbon-hydrogen data agree with those generally reported, but the methoxyl contents found are one-third to one-half the usual figures, corresponding to the lignins of young or immature tissues.

Table 4

Spectrophotometric Features of Gametophyte Extracts

Solvent	Species	Absorption Maxima (mμ) Direct Ultraviolet	Phloroglucinol Color (visible
Ethanol-HCl	Dawsonia	λ282	λ490,541
	Dendroligotrichum	λ280	λ487,540
	Polytrichum	-	-
	Psilotum	λ282	λ542
Ethanol-HCl from	Dawsonia	λ280	weak
H_2SO_4 residue	Dendroligotrichum	λ281	weak
1M NaOH	Dawsonia	λ300,318	λ542 (neutralized)
	Dendroligotrichum	λ295,317	λ544 (neutralized)
	Polytrichum	-	-
	Psilotum	λ301,321	λ540

Table 5

Analytical Characteristics of Moss Gametophyte Preparations

Sample	Sample wt. gm	Lignin mg/gm	Composition (%) C	H	OCH_3	Aldehydes (nitrobenzene) mg Hydrazone/100 mg Lignin
Dawsonia						
by H_2SO_4 (Klason)	0.500	85	61.2	6.8	7.9	47.4
by Ethanol-HCl	0.425	104	65.1	6.6	6.0	-
Ethanol-HCl	2.450	61	-	-	5.2	39.6
Dendroligotrichum						
by H_2SO_4 (Klason)	0.750	70	62.1	6.4	5.1	35.2

Lignin samples of 20-50 mg were subjected to the nitrobenzene oxidation. Their 2,4-dinitrophenylhydrazine precipitates were washed, dried, and weighed. If they were assumed to be the vanillin phenylhydrazone which is 40.3% vanillin, then 47.4 x .403 = 19.1 mg, or 19.1% of original lignin. If p-hydroxybenzaldehyde were the sole aldehyde isolated, contributing 37.8% of the weight of its phenylhydrazone, then the yielded would have been 17.9 mg, or 17.9% of the lignin sample. Although this latter assumption is ruled out by the substantial methoxyl content, either percentage value given falls into an acceptable range for the aldehydes liberated by nitrobenzene method.

According to Nord and DeStevens, "It has long been known... that early in the development of the cell walls of woody tissues of vascular plants there occurs a change whereby the cellulose is believed to become... what is generally described as lignified." This statement was set in the context of a brief account of the occurrence of lignin among the major subdivisions of the plant kingdom. Comparative chemical studies involving lignin in fossil plants have been carried out by Siegel, et al., and particularly by Manskaja, who reported vanillin-yielding residues in Devonian Pteropsids and, most significantly, vanillin-yielding moss remains from the upper Carboniferous. The paucity of information about lignin outside the Spermatophytes reflects the historical involvement of interest in these substances with wood, wood technology and the organic chemistry of natural products. Even phylogenetic interests have been in part outgrowths of interest in humus and coal. The other interest that has contributed to a knowledge of lignification involved development and differentiation. This aspect has also been concerned exclusively with angiosperms, however.

Although this inquiry was motivated largely by the hypothesis that extensive lignification of axial or other support structures may constitute a bio-mechanical adaptation, it also carries systematic, phylogenetic and comparative biochemistry into a new area.

Although one hundred thirty years have passed, since the discovery of lignins by Payen in 1838, it is still impossible to be both rigorous and concise when seeking to define them. Lignins thus seen stand in sharp contrast to other biopolymers--polysaccharides, proteins, nucleic acids. This apparent difficulty results from three points of difference between lignin and the other macromolecules: a), lignin monomers are covalently linked to one another in a variety of ways; b), there are a variety of monomers; and c), lignins are not subject to direct facile depolymerization.

It does not follow that lignins cannot be recognized, but rather that in any novel situation--as is the present one--a large number of tests must be applied in order to establish that an unknown is in fact a generic lignin. In essence, this is all that the writer has sought to accomplish, and this has been done along the following lines:

a. Solubility
    1. Solubility pattern in situ
    2. Differential solubility in situ and in vitro
    3. Decreased solubility of Klason preparation
    4. Ease of ethanolysis
b. Color Reactions
    1. Phloroglucinol, Cross and Bevan, Maüle tests
    2. Phenol tests

c. Chemical-Spectrochemical Properties

1. Ultraviolet absorption
2. Presence of 2,4-Dinitrophenylhydrazone-forming derivatives after ethanolysis ("hibbert Ketones")
3. Alkaline nitrobenzene oxidation to phenylhydrazone-forming products
4. Elementary and Methoxyl content

Although samples were insufficient to permit many other desirable tests to be completed including infra red spectrophotometry, hydrogenolysis and sodium scission in liquid ammonia, no other class of substances known conforms to the observations and analytical data reported here.

Lignin is therefore established as a significant constituent (up to 10%) of the moss gametophyte axis, but only in two cases involving gigantism, and not in Polytrichum, four other mosses and three liverworts. This lignin is largely conventional in its properties as far as they have been explored. The Cross and Bevan and Mäule tests indicate a lignin lacking the syringyl group. Although this group is typically absent in gymnosperms, the proportion of p-hydroxyphenyl-and guaiacyl- (or 4-hydroxy-3-methoxy-phenyl-) groups in them yields the average methoxyl content of about 15% and a vanillin content (from guaiacyl-) of 20-22%. The moss lignin contains only 5-8% methoxyl and yields perhaps 14-18% mixed aldehydes.

It is anticipated that further support among mosses and other cryptogamic plants for the proposed bio-mechanical basis for lignification will entail developmental analysis, biosynthetic measurements and experimental variations in mechanical and gravitational stress factors.

A-2 (a) Survival and Growth of the Larvae of the Mealworm Tenebrio molitar in Centrifugal Fields of 25-100 g.

This program is concerned with responses of plants to the absence of gravity. In order to proceed within a wider conceptual framework, hyper-g experiments were also carried out with plants and a few animal forms.

Relatively long-term experiments were conducted with the mealworm--over test periods of 5.5 months. Four to six week old larvae were placed on farina in plastic bottles on the centrifuge. At each concentric position on the centrifuge, a total of 300-500 animals were used. Exogenous water was not required as the larvae appear to meet their needs metabolically. Survival counts and weight data were taken at widely spaced intervals.

Control (1 g) survival ranged around ca. 95% throughout the test period. After 8 weeks survival was reduced somewhat at 25-50 g and appreciably at higher g-values (Fig. 2a-1). After 22 weeks, ca. 40% were alive at 25 g, ca. 12% at 50 g, ca. 3% at 75 g and none at 100 g. Although some 15-20% of 1 g animals initiated metamorphosis as pupae during the overall period, none of those under centrifugal stress showed signs of doing so.

Control animals weight gain over the test period was in excess of 150%. Even at 25 g, animals gained only 50% in 8 weeks, and lost weight at 75 and 100 g (Fig. 2a-2). After 18 weeks, only the 25 g group still persisted in weight gain. After 22 weeks, all groups had lost weight, but that loss was minimal at 25 g.

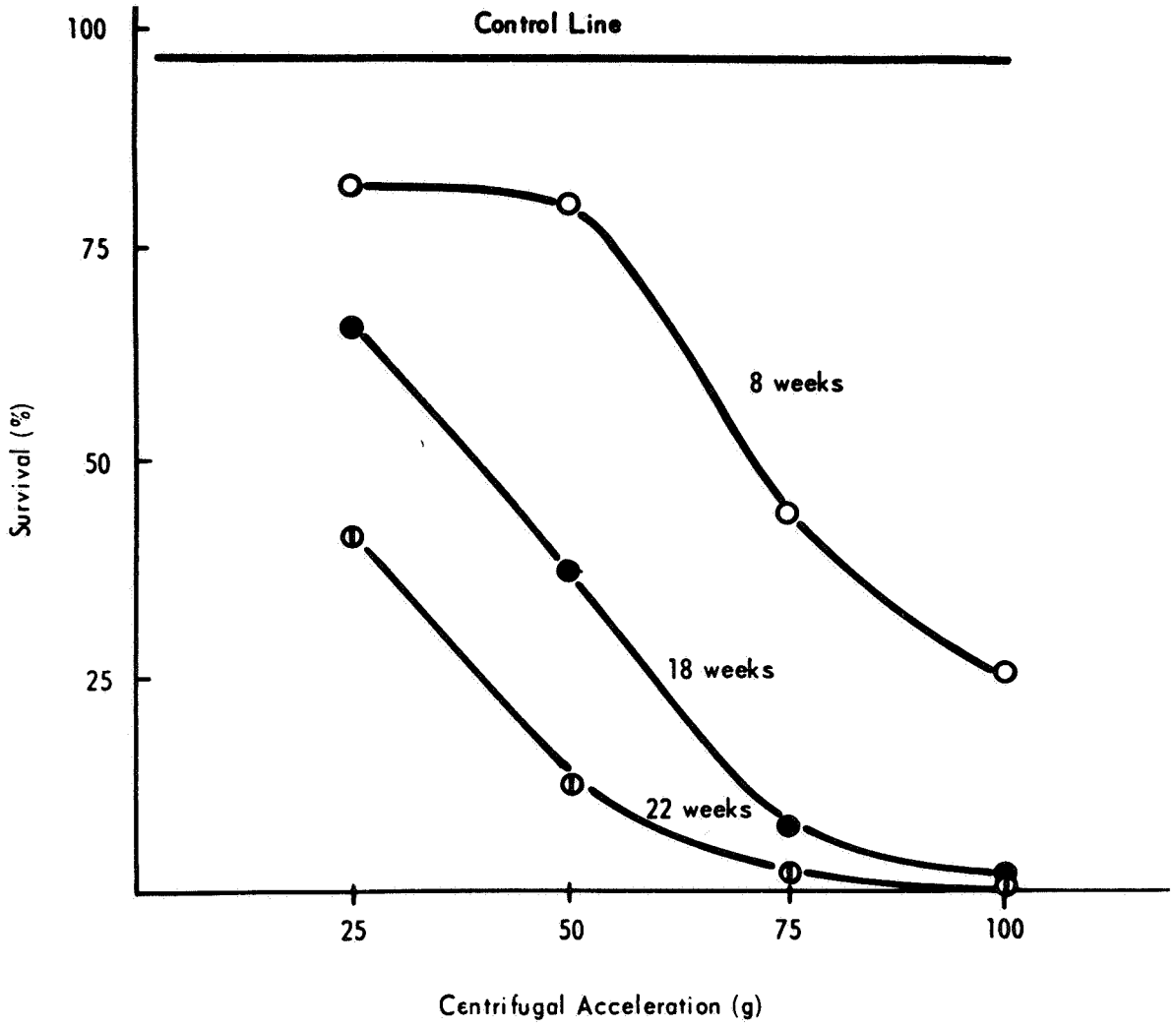

Fig. A-2a-1. Survival curves for Tenebrio larvae under centrifugal acceleration at 25-100 g.

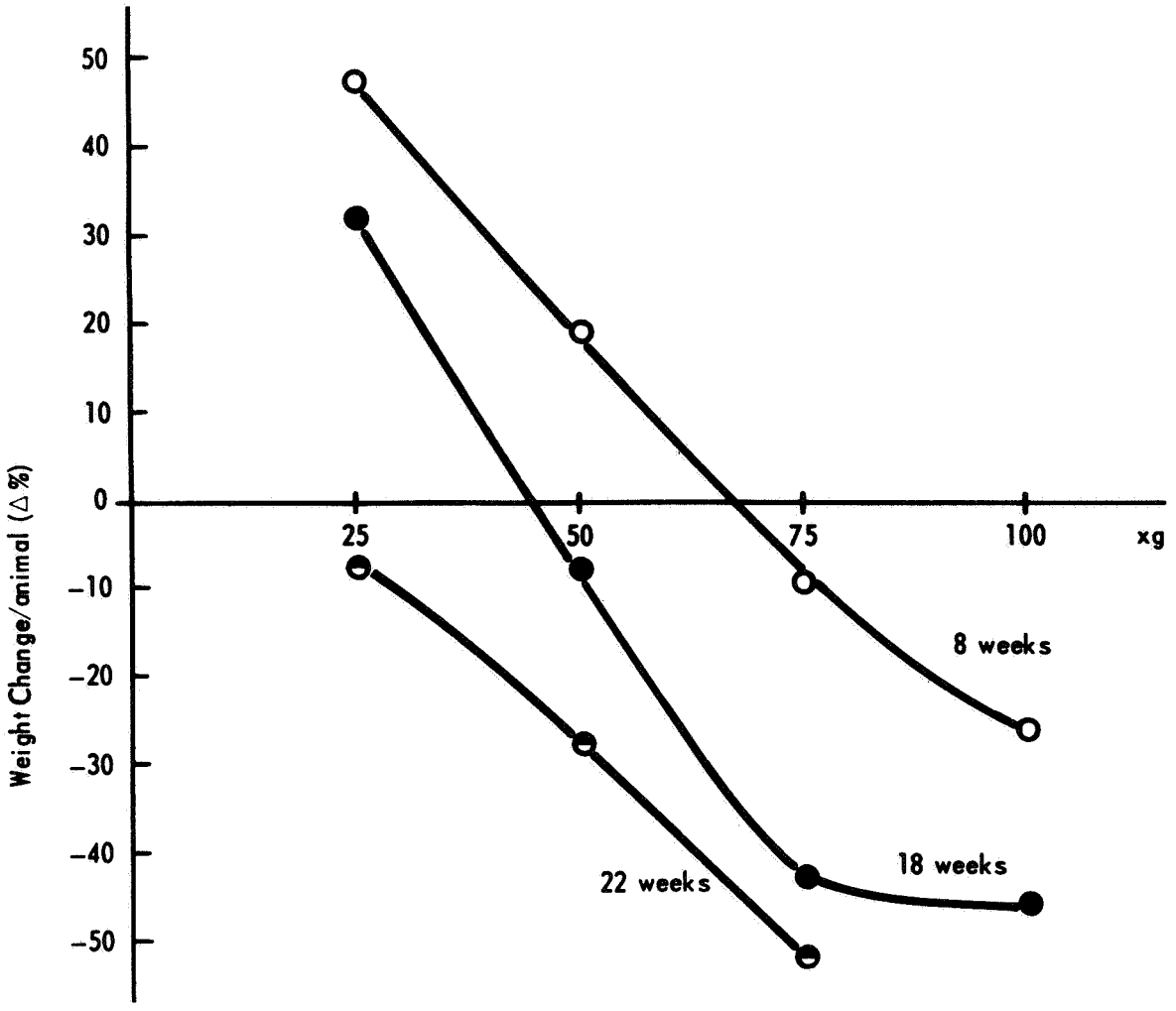

Fig. A-2a-2. Weight change curves as % initial value in Tenebrio larvae under centrifugal acceleration at 25-100 g.

A-2 (b) Seed Germination and Seedling Growth in Centrifugal Fields of 25-100 g

Seeds of mung bean, cucumber and winter rye were incubated at 24°C in polyethylene tubes. In each tube, 5 seeds were placed without substrate and just covered with water. In the first run, seeds were centrifuged at 25-100 g for 72 hrs.

Illumination was daylight fluorescent at ca. 200 ft-c for 12 hrs/24 hr the remainder being in darkness. Measurements after incubation showed suppression of germination and growth (Figs. A-2-b 1 and 2). Germination remained essentially at ca. 100% up to 50 g, but fell somewhat at 100 g; rye - 80%; cucumber - 71%; and mung bean - 38%. Only the latter species showed depression at 50 g-ca. 72% germination. Short (coleoptile) reduction in rye was observed, but was still persistant in most seedlings at 100 g. Sensitivity of radicles to hyper-g differed. Thus rye roots were inhibited completely at 50 g. Cucumber seedlings produced shorter radicles, but even at 100 g root growth was ca. 50% of its 1-g value. Mung bean radicles also held at a maximum inhibition less than 100%, ca. 20% of 1-g controls.

A better line of comparison is probably 50% inhibition of root growth:

	Interpolated g
Rye	13.5
Cucumber	24
Mung	16

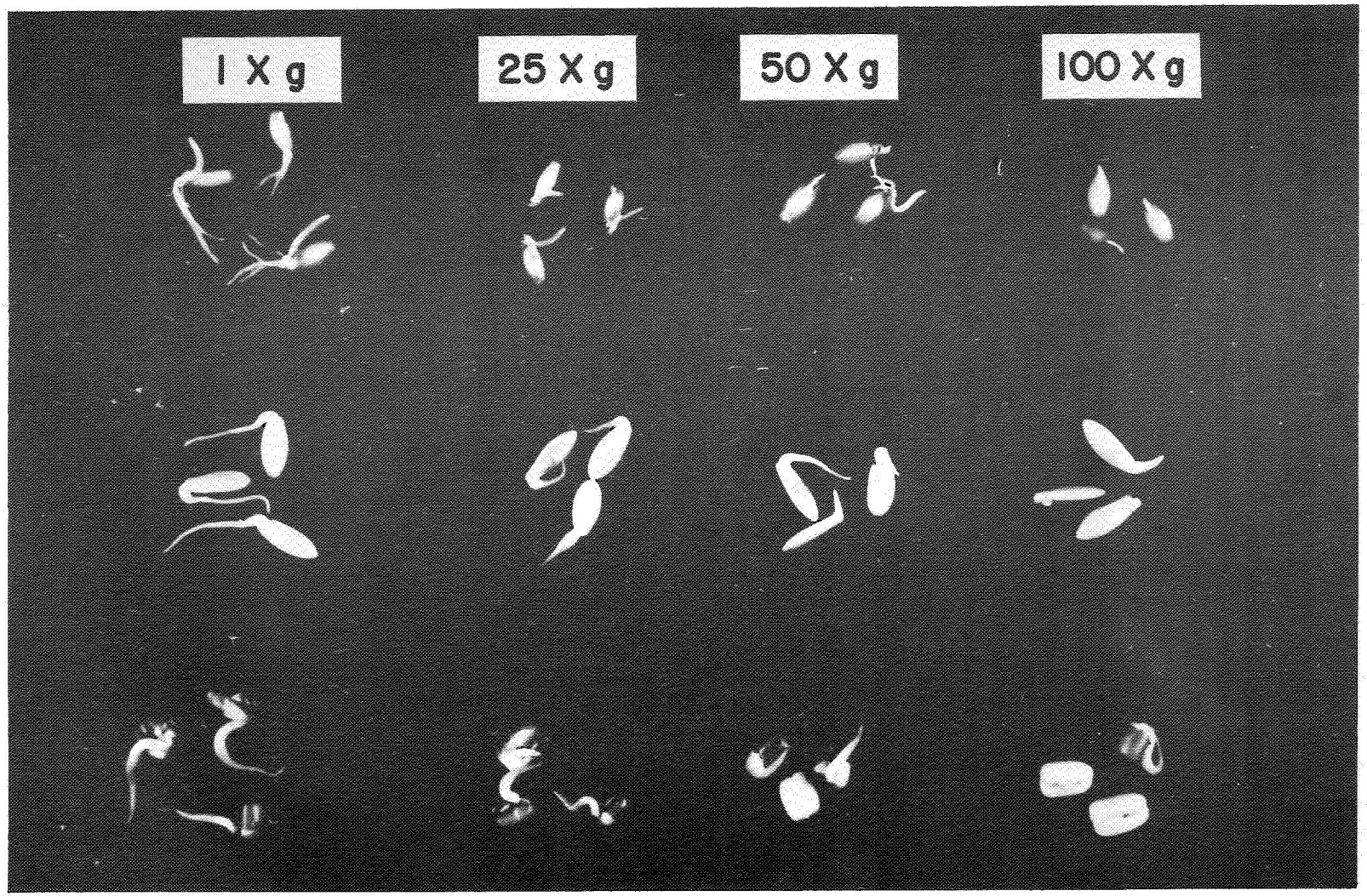

Fig. A-2b-1. Rye, cucumber, and mung bean seedlings after 72 hrs incubation.

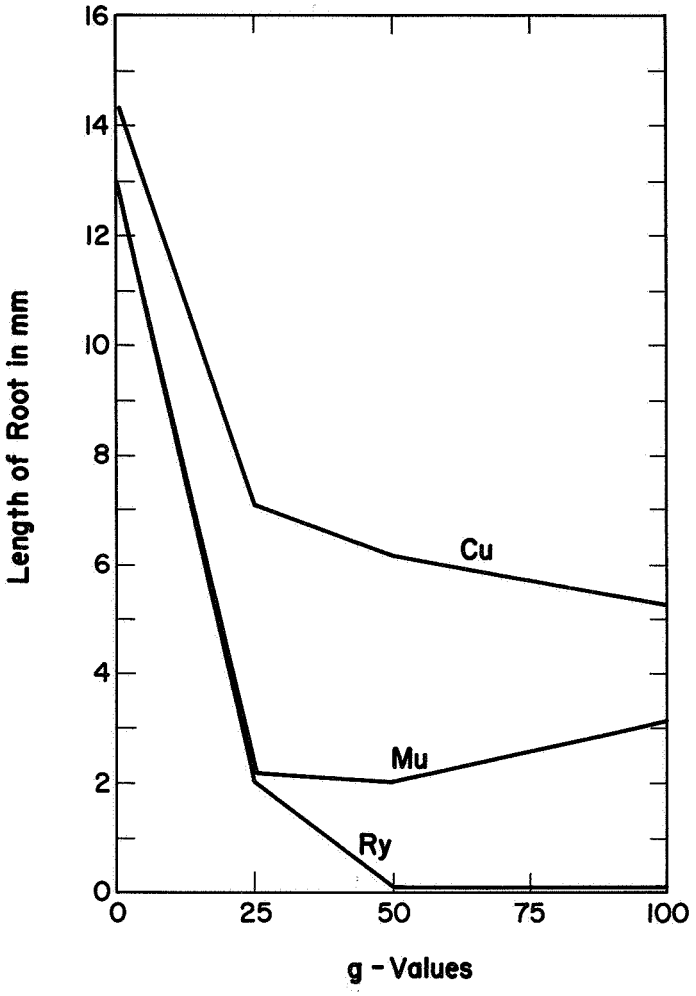

Fig. A-2b-2. Root growth as a function of g-value during 72 hrs centrifugation.

Although rye was most severly inhibited at 100 g, it was further studied in part because it lacks the resistance shown by the other species. Recovery from initial "shock" of 100 g seems to take place during continued incubation (Fig. A-2-b-3) as the relative figures show:

Incubation at 100 g	Relative Growth at 100 g (%)	
	Shoot	Root
3 Days	14	0
7 Days	40	51

Coleoptilar thickness is unaffected, although length is reduced, giving seedlings a stubby appearance. Pronounced curvatures are also characteristic of these hyper-g coleoptiles.

The dwarfing of these seedlings suggested a possible upset in hormonal relations. A few experiments were set up to test response to exogenous regulators. Some results, especially those with gibberellic acid (GA_3) were of interest, but experimental populations subdivided for dose-response measurements were too small and additional experiments are required. It is obvious that hyper-g stunting is a basic phenomenon in itself, and worthy of serious study.

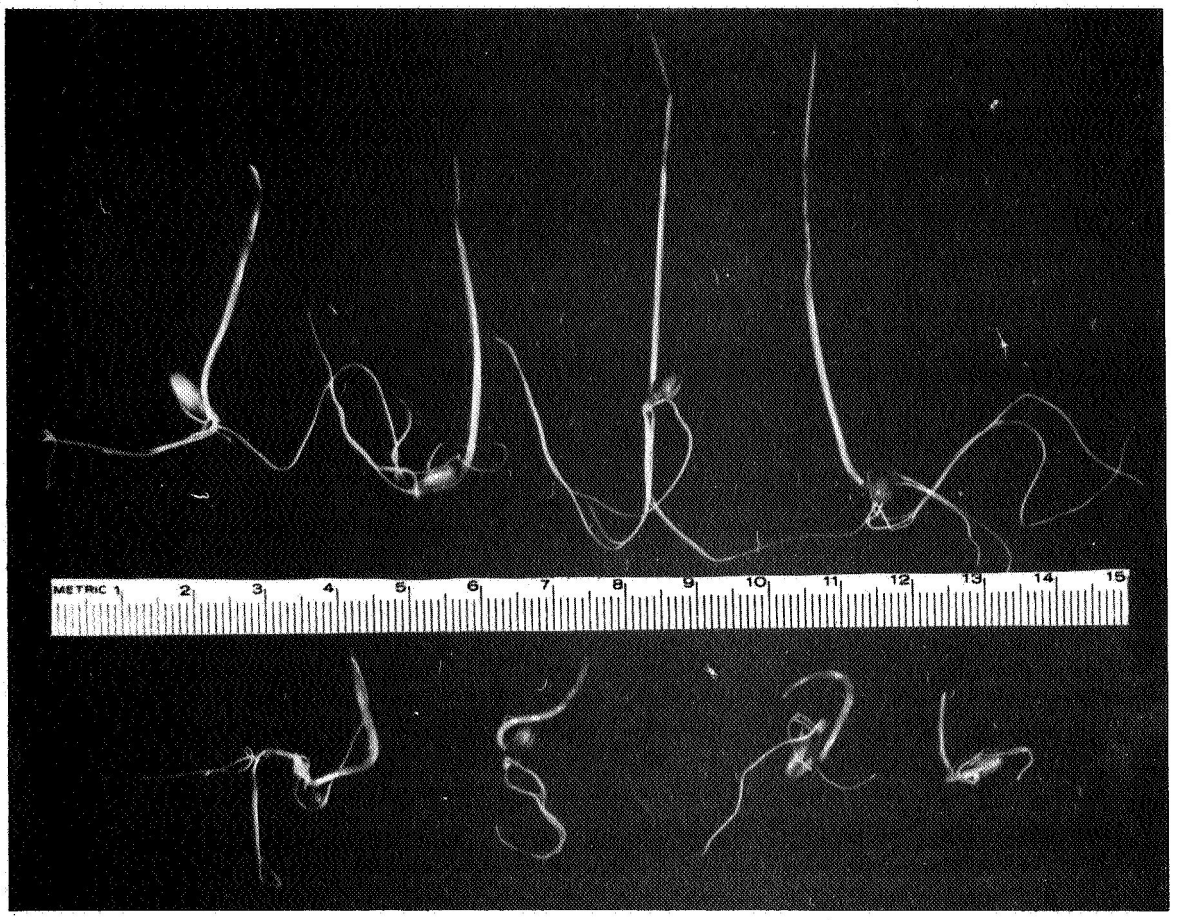

Fig. A-2b-3. Comparative growth of rye seedlings for 7 days at 1 g and 100 g. Above rule - 1-g controls. Below rule- 100-g plants.

A-3 Experimental Induction of Lignification in Mangrove Seedlings (Rhizophora mangle)

Seedlings of the mangrove (Rhizophora spp, principally R. mangle, fig. A-3-1) are produced from embryos released from the parent tree free of cotyledons and maternal tissues. With an aerodynamic form and low center of mass, they tend to land upright imbedded to a depth of perhaps one centimeter in the muck of their swamp habitat. During studies concerned primarily with effects of salinity and O_2 on growth of the seedling it was observed that slices from the starch-packed hypocotyl, which comprises the bulk of the young plant, became pink, then brown, almost instantaneously in air, but not under N_2. Rapid transfer to cyanide solutions or boiling water also retards tissue discoloration.

There appears to be present in mangrove both a phenol oxidizing system and a sizeable supply of phenolics. In addition to leuco compounds, there are epidermal and hypodermal cell layers rich in an anthocyanin readily extracted with HCl-ethanol. This compound has not been identified, but has a color quite similar to cyanidin. Use of the phloroglucinol test for lignin is rendered quite difficult by the intense red color appearing in HCl-ethanol, but the Cross and Bevan reaction-generation of a red color in lignins by treatment with chlorine then NH_4OH or Na_2SO_3 solution--can be carried out in the presence of phenolics, including flavonoids, without interference.

It was found that the mangrove hypocotyl yielded at best, a diffuse pale red color when sliced and run successively through HCl-5% NaOCl (15-18 hrs), water rinse, and Na_2SO_3 (sat'd aq.) or

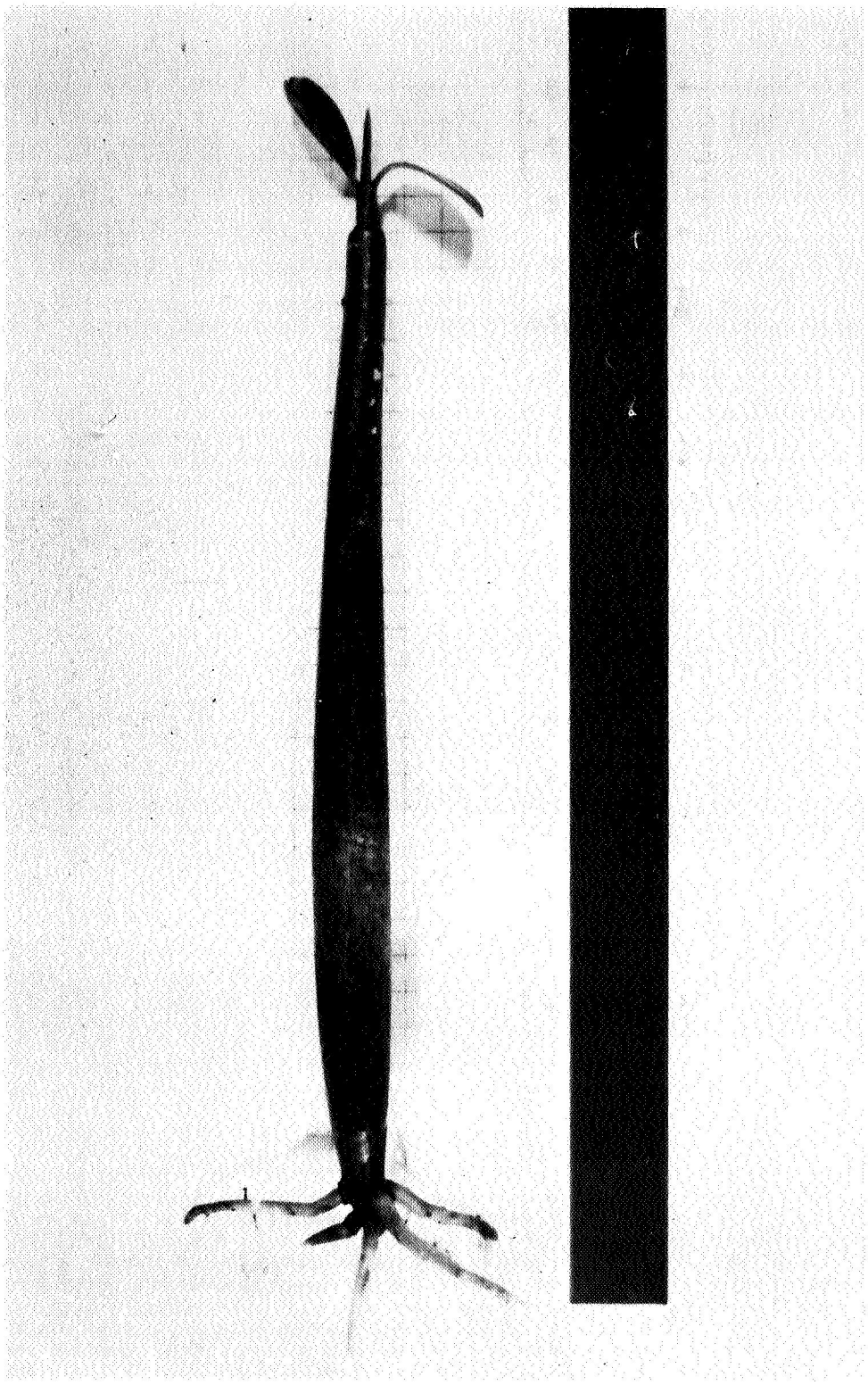

Fig. A-3-1. Young mangrove seedling several weeks after being shed.

NH_4OH (5 min). In contrast, sections taken through specimens of Rhizophora bearing wood-like necrotic lesions on the hypocotyl typically showed a dense positive red color around and within the woody tissue, but not in tissues several centimeters away from the necrotic zone. Thus it was suggested that although mangrove seedlings eventually become sizeable trees, the virtually unlignified juvenile stage itself can be induced to form localized centers of lignification as a result of infection or other biopathic states. Further evidence for this comes from a general phenomenon well known to phytopathologists of isolating infection loci--in leaves for example--by laying down a circular wall of quinonoid compounds and lignin.

Mangroves 6-9 inches long can hold ca. 0.5 ml water, injected via hypodermic. When water alone is injected, within ca. 10 days a strong lignin color can be developed by Cross and Bevan treatment immediately around the puncture (Fig. A-3-2) and not elsewhere. From a consideration of this finding and the situation created around infection centers, additional seedlings were injected with either 0.5 ml of 10^{-4}M poly-l-lysine, or 0.5 ml of 10^{-3}M coniferaldehyde.

Again, within 10 days, Cross and Bevan color patterns were developed. They showed that polylysine induced an intense, widespread reaction whereas the lignin precursor produced a moderately strong positive reaction zone surrounded by a less intense zone suggestive of a diffusion pattern.

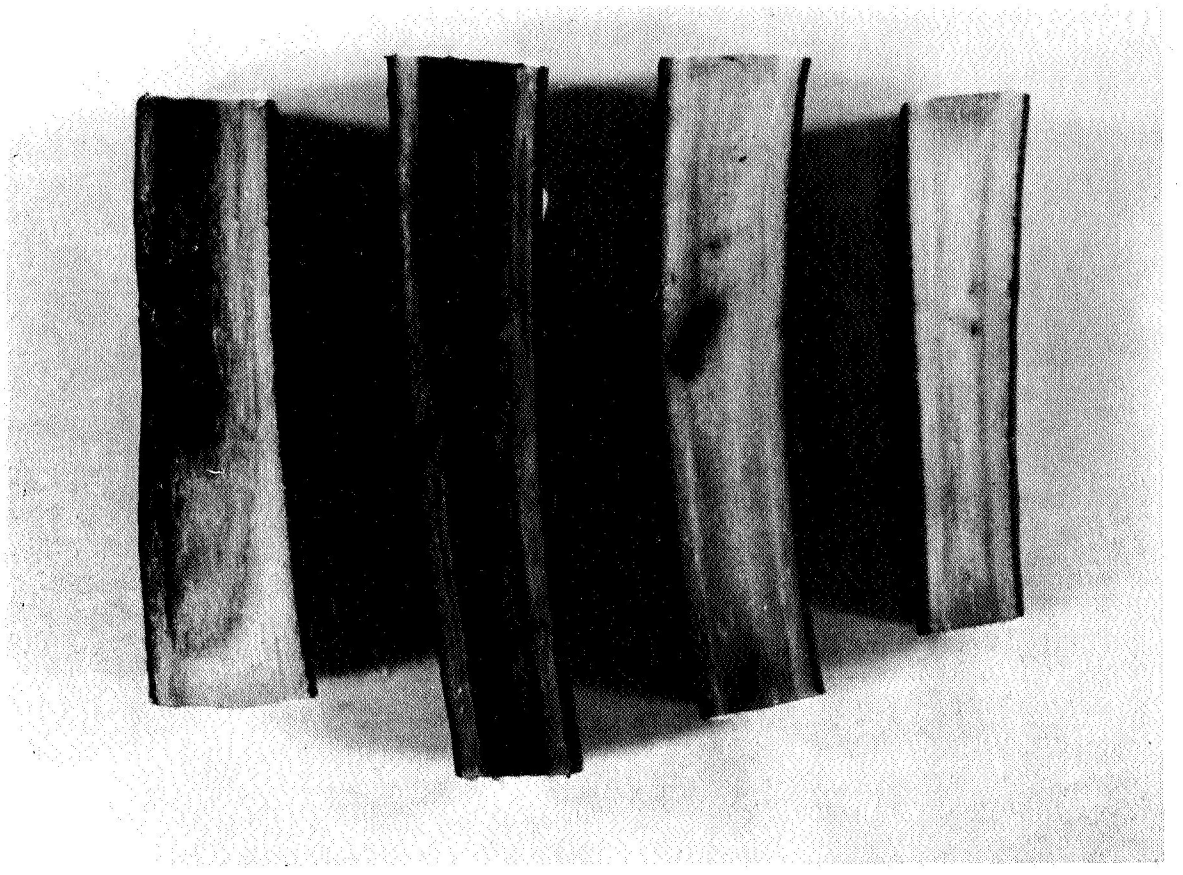

Fig. A-3-2. Cross and Bevan color reaction on mangrove slices. Left to Right: Control plant; water-injected; polylysine injected; coniferaldehyde-lysine.

The striking effect of polylysine warrants special comment. Polylysine was discovered by the investigator to increase membrane permeability and has been discussed previously in connection with our interest in membrane function as a factor in stress resistance. It appears to operate by displacement of membrane Ca-ion.

These data suggest that membrane permeability may be a limiting factor in lignification in mangrove, and that either an increase in permeability or an actual membrane breakdown may initiate some essential step in the process. These membrane changes may be brought about by chemical means, mechanical disruption, or microbial action, or presumably, in the course of normal cell maturation and senescence. Further, it is not evident whether we are considering the cell membrane itself or, more probably internal limiting membranes providing compartmentation of lignin-determining biochemical steps or reactions.

The next phase in this study was the exposure of seedlings to hyper-g conditions in the centrifuge (Fig. A-3-3). After 2 weeks at 25 and 75 g, plants were removed, sectioned longitudinally and treated for lignin color development. From the observations made, it may be concluded that an artificial gravitational stress can induce lignification (Fig. A-3-4). Pre-extraction of the tissues with hot water and hot ethanol failed to alter the pattern. Indeed, such procedures sharpened the color differences by removing small soluble chromogenic molecules.

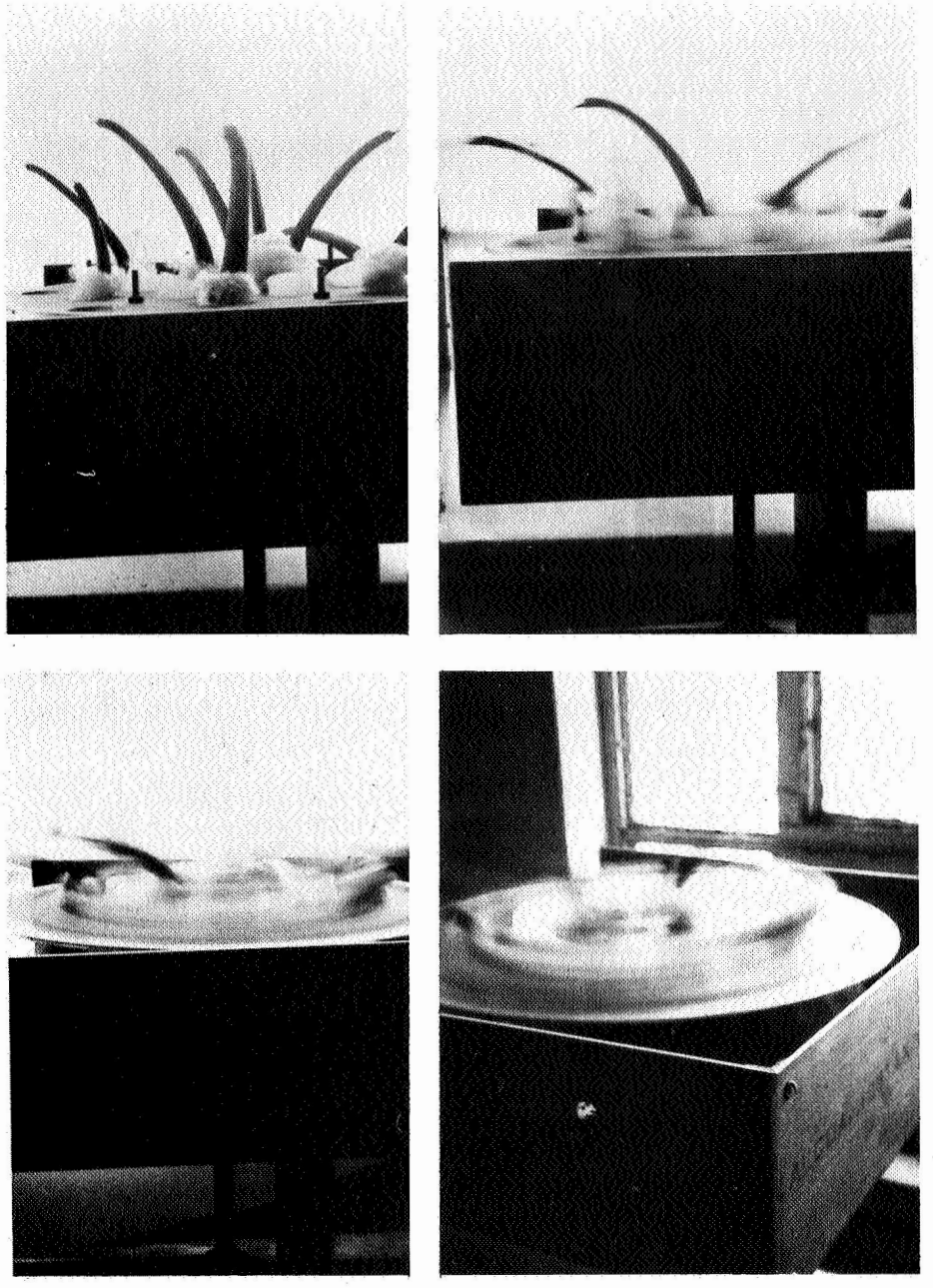

Fig. A-3-3. Centrifugation of mangrove seedlings. Upper Left to Lower Right: acceleration of seedlings from stop to full speed.

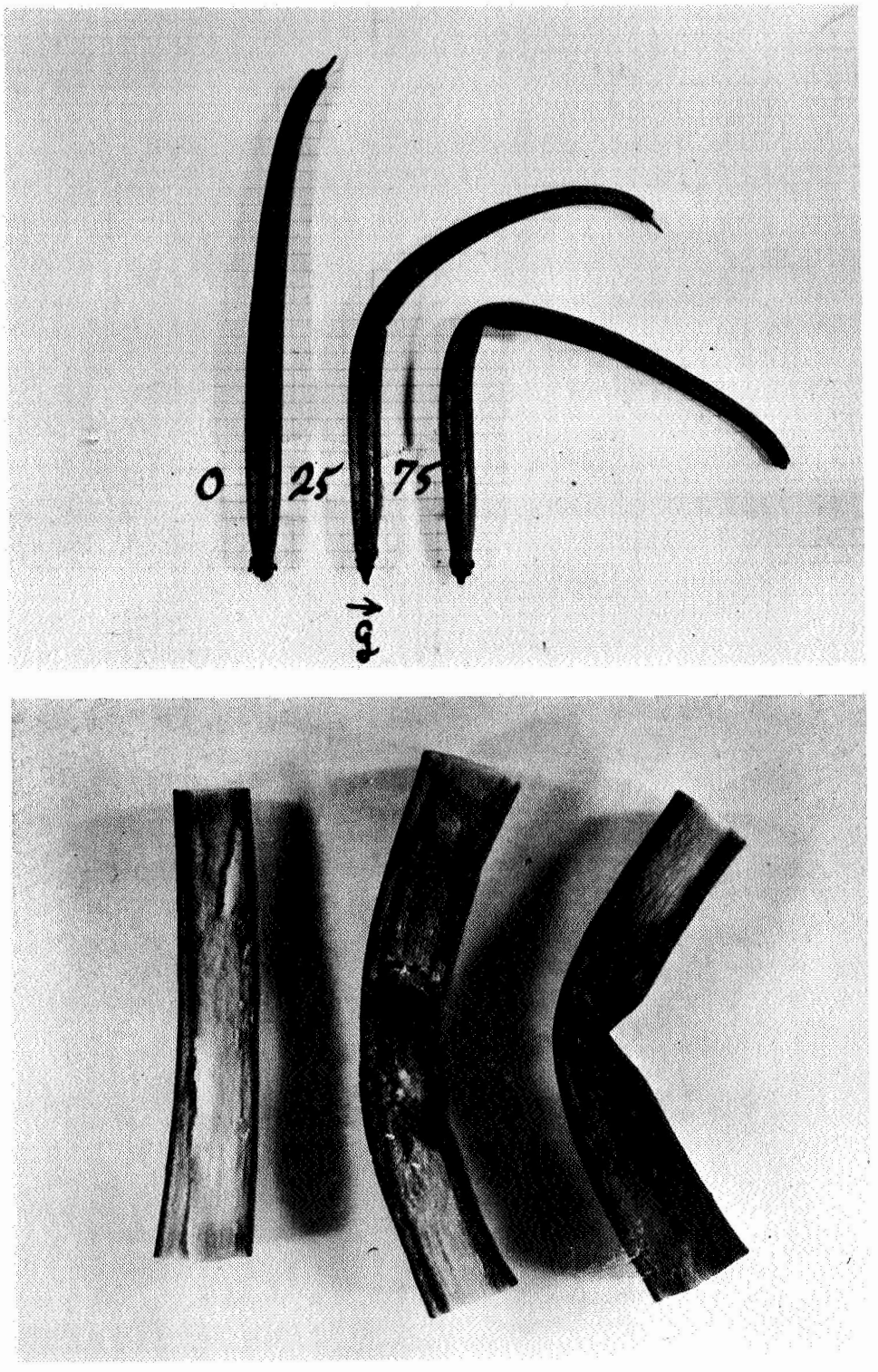

Fig. A-3-4. Effect of 2 weeks of 25-15 g upon Cross & Bevan color for lignin. Above: Conformation of plants; Below: induced color reactions in seedling slices.

If the categories of experiment described are taken together, it appears possible to equate some of the effects of compression forces with those of mechanical and chemical alteration in permeability and microbially induced necrosis, thereby suggesting that gravitational-mechanical stress may operate to set in motion a chain of biochemical events by acting initially upon membrane permeability. Logically, there must be genetic and perhaps developmental determinants of the ability to become lignified. Elodea, an aquatic angiosperm has lost its ability to become lignified because it cannot make precursor hence is genetically incapable of such responses. When mature Coleus plants which have well developed vascular bundles are subjected to extreme deformation by tying branches back on themselves (Fig. A-3-5). There is no indication of enhanced lignification. These plants will regenerate xylem when it is cut experimentally, but does so in an already determined geometric pattern. Perhaps the juvenile character of the mangrove was important in predisposing the plant to the kinds of changes noted here.

Even given the unrepressed genetic capabilities and suitable stage it does not follow that the membrane deformation hypothesis can be applied to the 0 to 1 g range as it appears to under hyper-g conditions. It is however a possibility well worth investigating.

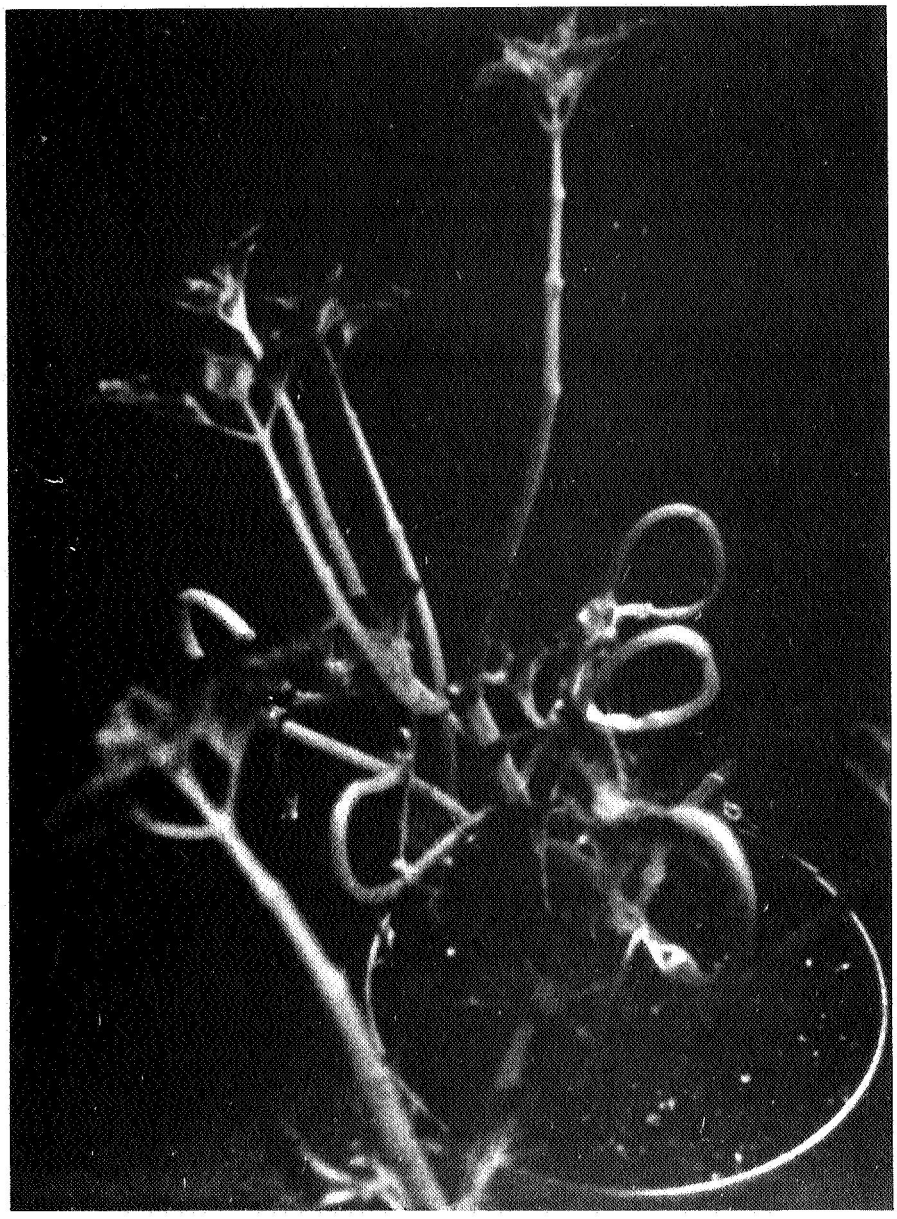

Fig. A-3-5. Mechanical treatment of Coleus branches: An effort to study induced patterns of lignification.

B. INITIAL EXPERIMENTS WITH HYPO-G SIMULATION USING CUCUMBER SEEDLINGS

1. General Growth Characteristics of Cucumis sativus var. Black Diamond

Many years of experience have shown cucumber to be a highly reliable experimental plant for growth studies. Seed germination is rapid and seedlings grow with little care or maintenance in media such as vermiculite for periods of at least two weeks. Cucumber seeds do not support growth of fungi or bacteria as readily as seeds of many leguminous and cereal species used in the laboratory. Initial increase in axial fresh weight (root + hypocotyl without cotyledons) and total length (hypocotyl tip to primary root tip) is exponential (Fig. B-1-1). Dry matter content of the axis tends to rise, then fall whereas lignin content shows a stepped rise. This lignin determination was carried out by the Klason 72% sulfuric acid method which tends to yield high values in young tissue by means of its dehydrating effect on polysaccharides. As will be seen below, analytical methods for lignin have been given further consideration.

Other features of cucumber seedling growth of value include tolerance to a wide pH range--ca. 3 to 11--and to moderate salinity, ca. 1% NaCl.

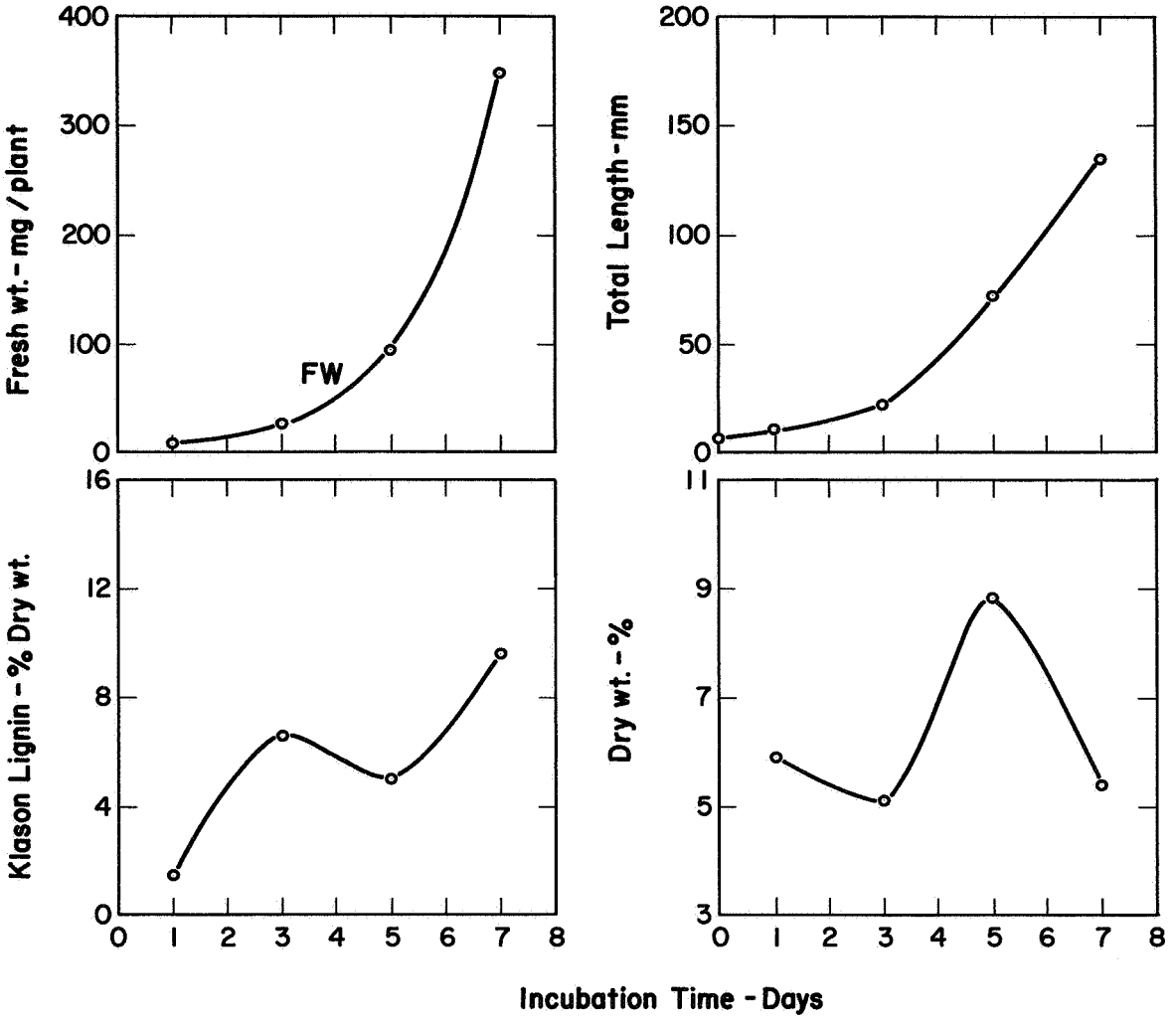

Fig. B-1-1. Some characteristics of growth in young cucumber seedlings. Upper left--fresh wt. of axis (hypocotyl + root without cotyledons); Upper right--total length of axis; Lower left--Klason lignin; Lower right--dry weight.

B. INITIAL EXPERIMENTS WITH HYPO-G SIMULATION USING CUCUMBER SEEDLINGS

2. Experimental Hypo-g Regimes: Aquatic media and the clinostat

The Klason technique for lignin determination tends to give erroneously high values, but in comparisons run between the Klason method and a quantitative adaptation of the well known phloroglucinol (or Wiesner's) test, a satisfactory correspondence was established.

The phloroglucinol test was first converted to a quantitative photometric procedure in 1960 (Plant Physiol. 35, 163-167). In the present modification, oven-dried tissue is first pre-extracted in hot 95% ethanol for 30 min. ca. 25 ml per 0.1 gm dry matter then in hot 3% HCl in ethanol. The pre-extraction with neutral ethanol removes monomeric precursors that might interfere with the color reaction.

To the acid-ethanol extract after cooling is added an equal volume of 1% phloroglucinol in ethanolic HCl 1:1 by volume and the optical density (O.D.) determined at 540 mμ after 30 seconds. The conversion of density readings to lignin (as % of dry wt.) was based on the satisfactorily low Klason readings -- all under 5% -- and reasonable correlation with the phloroglucinol method which has an independent basis. For any sample the following relationship was established:

$$\frac{\text{O.D. x ml extract x 0.5}}{\text{gms of dry wt. extracted}} = \text{lignin as \% dry wt.}$$

Three variant forms of aquatic technique have been tried, but they have not yet been set up on a strictly comparative fashion. These procedures include <u>static aquarium</u>, wherein plants grow from seeds fixed in position, aeration being accomplished without disturbance; the <u>slow current aquarium</u>, with water moved by the aeration stream (Fig. B-2-1 and B-2-2); and the <u>high turbulence</u> system effected by forcing air from

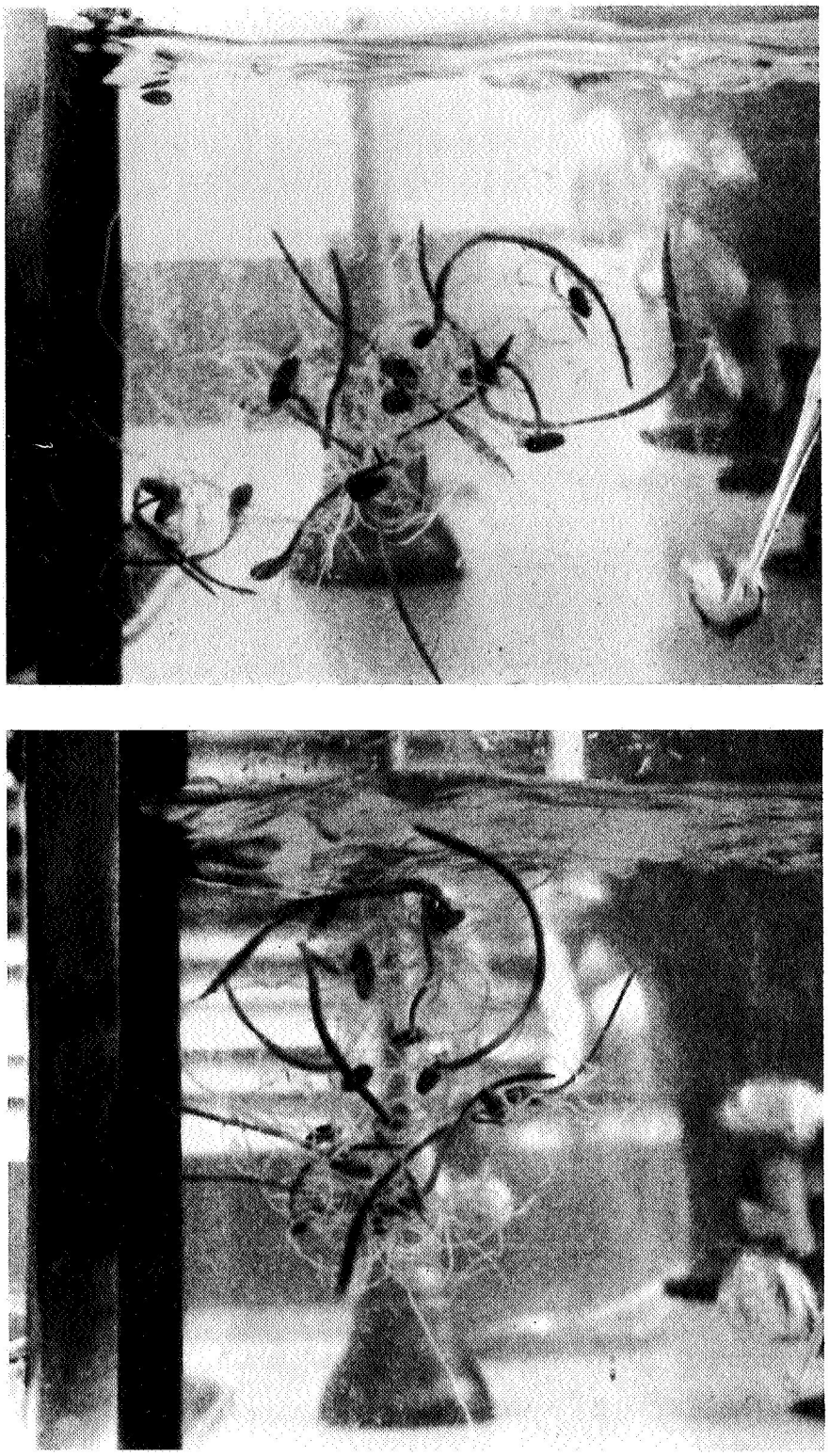

Fig. B-2-1. Two pictures of winter rye seedlings in the slow current aquarium taken at 30 seconds showing extent of movement.

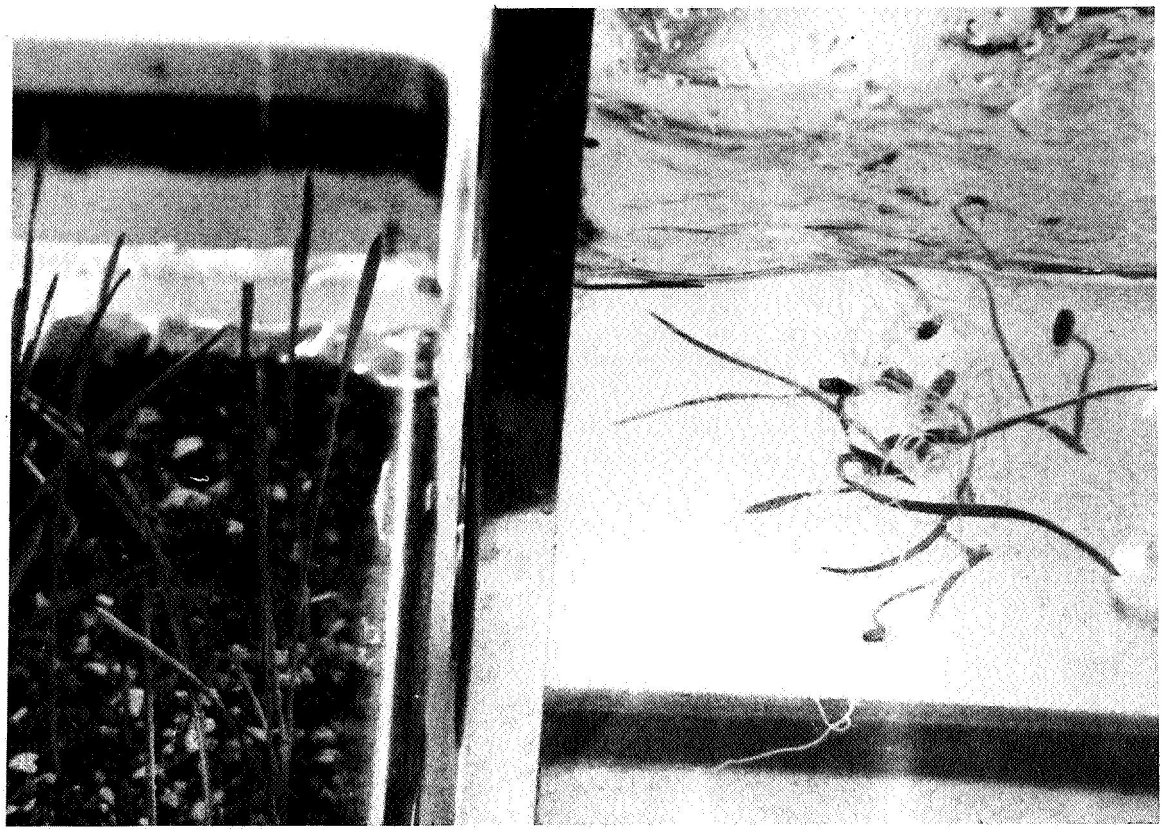

Fig. B-2-2. Ten day old flat-grown and water-grown rye seedlings compared.

beneath through water contained in an ordinary 1-3 liter separatory funnel. Seeds, and subsequent seedlings are subjected to continuous vigorous motions yet are permitted no net orientation. Growth is profoundly affected by this technique (Fig. 3-2-3).

One variant form of the static culture deserves mention as a hitherto untried method; namely the liquid-liquid system (Fig. B-2-4). Some seeds can be germinated and seedlings grown supported at a liquid-liquid interface provided that the upper layer is gently aerated and the underlayer is immisable with water, and higher in specific gravity.

Within the limitations imposed by morphology and experimental conditions aquatic media affect root orientation in a manner consistent with a reduced gravitational stimulus (Table B-2-1). The response may involve frequency of negative geotropism or root-hypocotyl angle. The latter eventually approaches 180° in conventional culture, but never exceeds 90° greatly in aquatic media. Cucumbers cultured on the experimental clinostat (Fig. B-2-5) show the same angular root-hypocotyl relationship found in aquatic cultures.

With respect to growth patterns (Table B-2-2), ordinary flat-cultured seedlings (vermiculite) were compared with plants grown in gas phases of 5% O_2 and 1% O_2 respectively. Consistent with earlier work summarized by Siegel et al., (in Briggs and Mamikunian, eds "Current Aspects of Exobiology", JPL Report publ. by Pergamon Press, 1965) cucumber seedlings grown in 5% O_2 are longer and heavier than those grown in air. In 1% O_2, growth is O_2-limited. Aerated water contains, at saturation, ca. 0.8 vol. % O_2 and oxygenated water, ca. 4 vol % O_2. These figures are close enough to the gas phase levels to permit comparison: -- oxygen aquatic

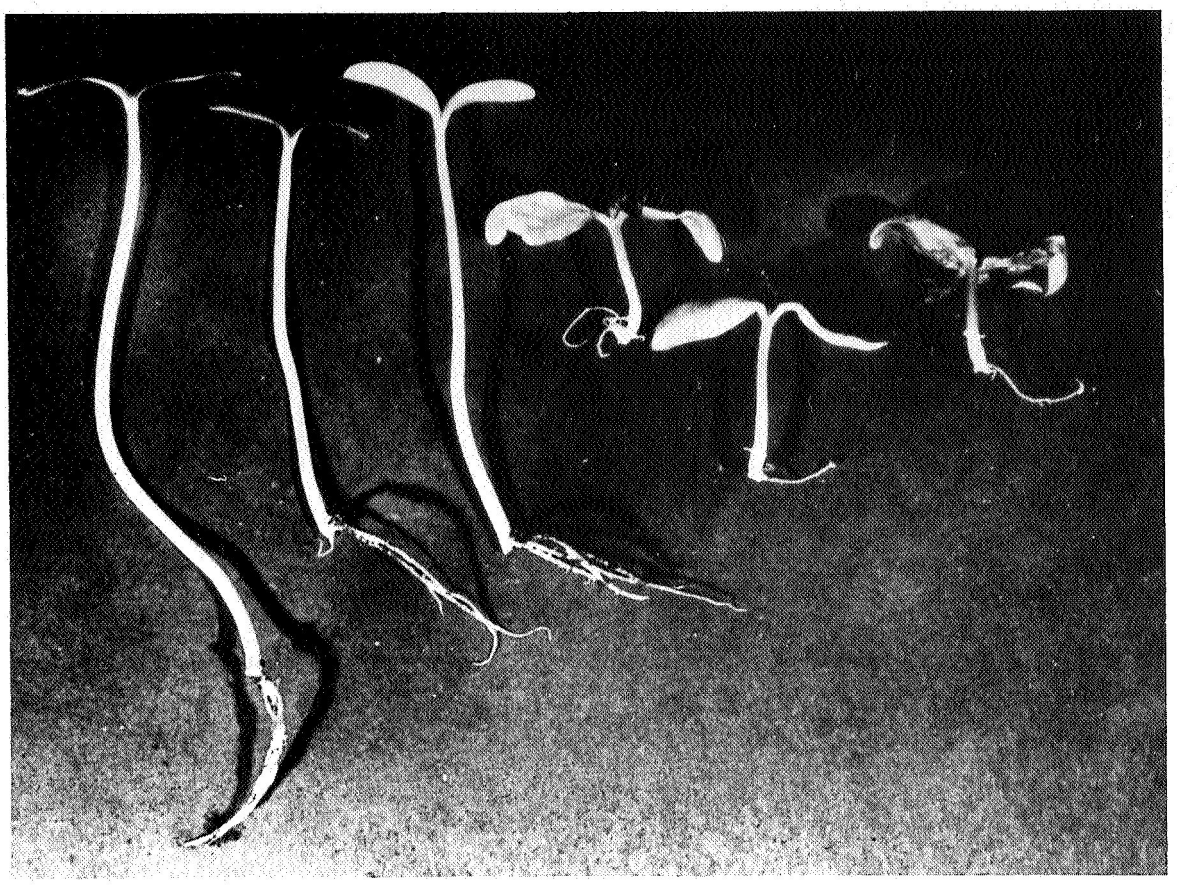

Fig. B-2-3. Cucumber seedlings at 10 days. Left: flat grown in vermiculite. Right: growth in turbulent water.

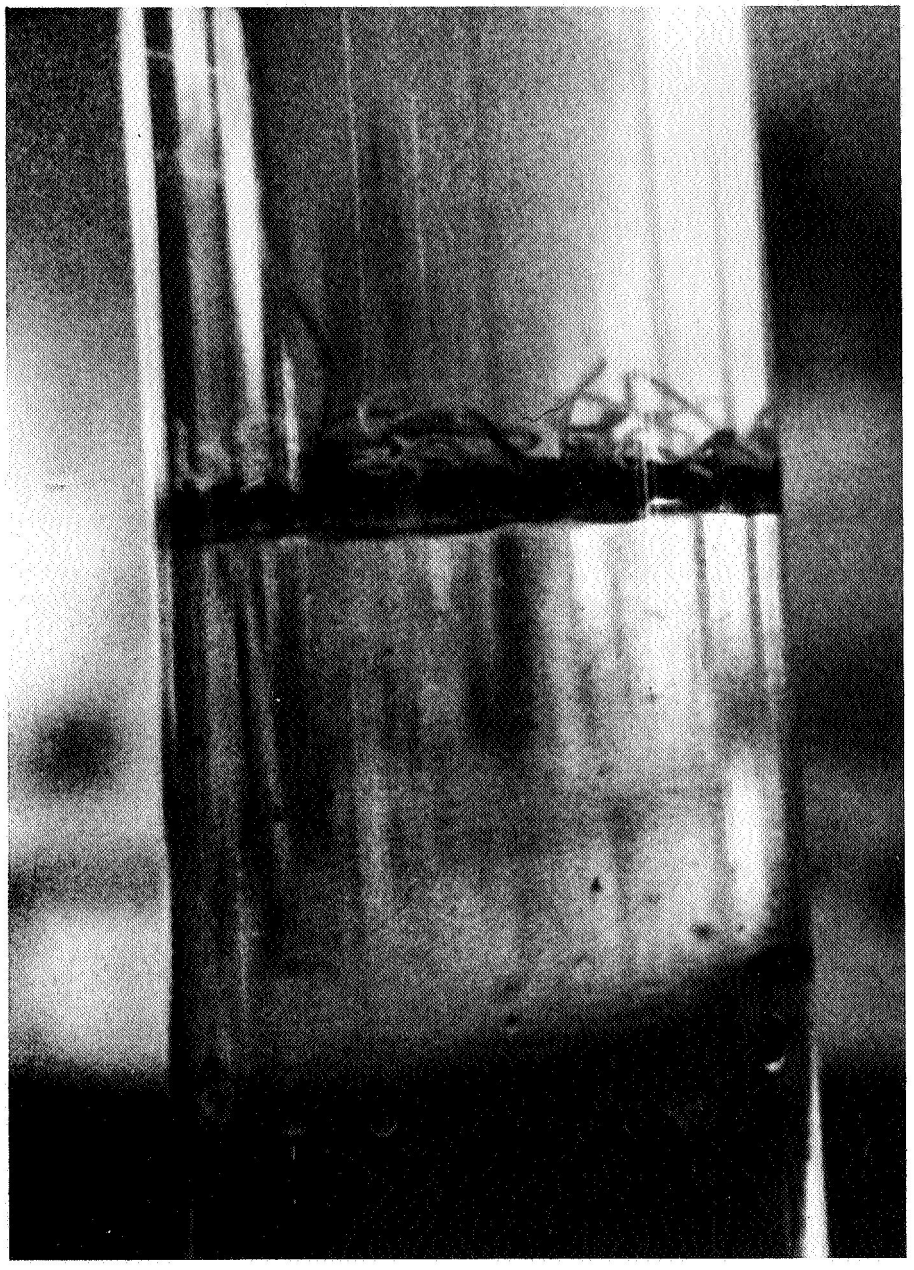

Fig. B-2-4. Onion seedlings 7 days old grown at the interface between water and Dow fluorocarbon. The latter has on O_2 solubility ca. ten-fold greater than water, but this is not directly relevant to the experiment.

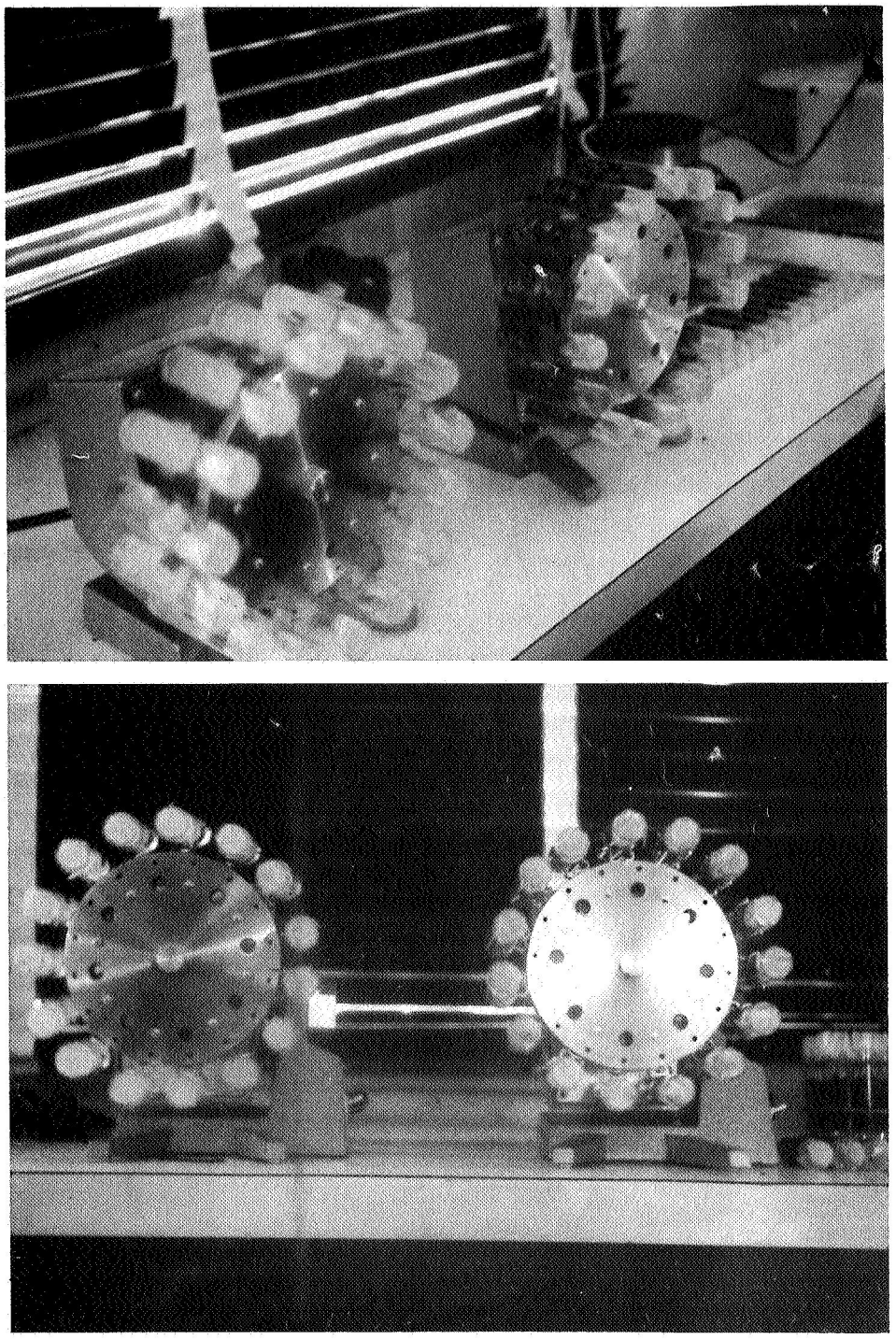

Fig. B-2-5. Views of experimental clinostats with culture vials attached. Given— $g_{rel} = W^2 r\, g^{-1}$ where $g_{rel} = ag^{-1}$. W = angular velocity in rev. x sec^{-1} and g = 980 cm x sec^{-2}. In these clinostat experiments r = 9 cm and W = 0.11 rev. x sec^{-1}, then $g_{rel} = 1.1 \times 10^{-4} g$.

Table B-2-1

A Comparison of Root Orientation in Aquatic and Conventional Media

Cultured Plant	Medium	Orientation	
		Root-Hypocotyl Angle	Negatively Geotropic Roots (Est'd %)
10 Day Winter Rye	Vermiculite	-	0
7 Day Onion	Vermiculite	-	0
10 Day Boussingaltia	Vermiculite	-	0
7 Day Cucumber	Vermiculite	135°	0
10 Day Winter Rye	Static Aquarium	-	25
7 Day Onion	Liquid-liquid interface	-	45
10 Day Boussingaltia	Static Aquarium	-	15
7 Day Cucumber	Static Aquarium	88°	12
	Slow Current Aq.	91°	-
	High Turbulence Aq.	97°	-
	Clinostat 1	ca. 90°	-
	Clinostat 2	ca. 90°	-

Table B-2-2

A Comparison of 7-Day Growth Patterns

for Cucumber on Experimental Regimes

Medium	Length (mm)		Fresh Wt (mg)		Dry wt (mg)	
	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl
Vermiculite (air)	82±9	107±12	137±18	190±24	6.1±1.1	6.0±1.0
Vermiculite (5% O_2)	110	204	195	240	5.6	7.2
Vermiculite (1% O_2)	6	-	5	-	0.3	-
Slow Current Aq/air	75	58	77	165	2.8	5.3
Slow Current Aq/O_2	55	85	85	210	1.1	6.0
High Turbulence Syst.	21	20	40	100	1.9	3.3
Clinostat 2	31	45	120	120	29.4	4.3
Clinostat Controls						
Vertical Static	98	80	80	210	13.4	7.0
Horizontal Static	85	61	70	140	14.9	5.4

vs. aerated aquatic, 5% gas phase vs. oxygenated aquatic and 1% gas phase vs. aerated aquatic. In most respects, growth parameters in 5% gas phase plants exceed those in the oxygen aquatic regime, whereas the opposite is markedly the case when the lower oxygen levels are compared. Thus 1% O_2 cannot support the growth of cucumber seedlings at an appreciable rate into the gas phase, but can do so when supplied in solution in a buoyant medium.

With respect to O_2 supply, aerated aquatic media, whether slow current or turbulent type should have been equivalent. Mechanically the two regimes are of course quite different, and every growth parameter shows this difference in terms of marked reduction in the turbulent systems. Nevertheless, the seedling produced after 7 to 10 days of culture is sturdy and deep green (see Fig. B-2-3).

A consideration of the growth pattern yielded on the clinostat must first center about proper controls. Although flat-grown seedlings can be used in a sense as ultimate controls, the fact of deliberate orientation requires that the horizontal static cultures be used as primary standards for comparison (Fig. B-2-6).

The two general regimes used in these experiments both involve hypo-g components operating for somewhat different reasons. The aquatic media offer a buoyancy factor whereas the clinostat makes possible the summation of g-forces uniformly around the main axis of growth. In addition, the aquatic medium can be used to randomize the orientation of suspended plants. It is not possible to judge the respective merits of limitations of these procedures at present, and will only be subject to a comprehensive critical analysis when comparisons can be made with in-flight data.

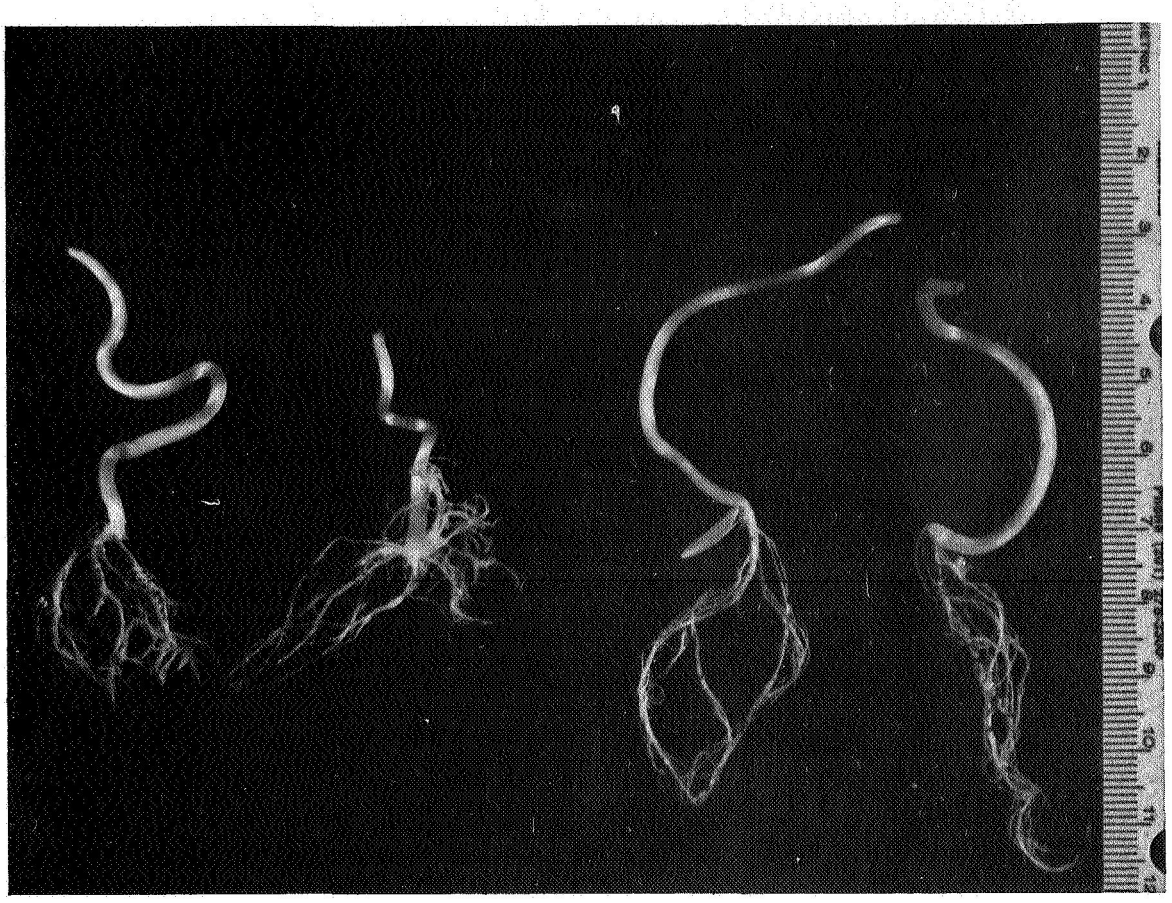

Fig. B-2-6. Axes of cucumber seedlings from the clinostat (left) and horizontal static groups (right) after 7 days.

Certain relationships between lignin and experimental conditions are of particular interest (Tables B-2-3 and B-2-4, Fig. B-2-7).

(a) In the gas phase, hypocotyl lignification is markedly O_2 limited, whereas root lignification capacity is saturated at ca. 5% O_2.

(b) Growth of both organs seems to pass through an optimum at 5% O_2.

(c) At the higher O_2 levels, hypocotyl lignin and linear extension are inversely related, a relation barely evident in the root.

(d) In aquatic media, both root and hypocotyl lignification are O_2- united. Here root growth and lignification are inversely related whereas hypocotyl growth and lignin content are not.

(e) Lignification is reduced in aquatic media relative to vermiculite (air), particularly in the hypocotyl.

(f) Although lignin is also reduced in the turbulent system, the reduction is not as great as in the slow current medium. This may reflect a response to mechanical stimuli of a turbulent system.

(g) Relative to comparably grown seedlings in vermiculite vials, lignification is reduced on the clinostat, but more so in the root than hypocotyl. The most valid reference is presumably the horizontal vial.

Table B-2-3

A Comparison of Lignin Contents of 7-Day Cucumber Seedlings on Experimental Culture Regimes

	% of Dry Wt.	
	Root	Hypocotyl
Vermiculite (air)	1.66±0.31	1.97±0.42
Vermiculite (5% O_2)	1.52	0.88
Vermiculite (1% O_2)	0.63	-
Slow Current Aq./Air	1.21	0.91
Slow Current Aq./O_2	1.65	1.01
High Turbulence Syst.	1.34	1.08
Clinostat 2	0.34	2.42
Clinostat Controls		
Vertical Static	1.30	3.03
Horizontal Static	1.00	3.72

Table B-2-4

Calculated Δ% Lignin under Experimental Culture Regimes

	Root	Hypocotyl
Slow Current Aq. (Air)		
vs % Vermiculite (Air)	-27	-53
vs % Vermiculite (1%)	+92	-
Slow Current Aq/O_2		
vs % Vermiculite (Air)	-1	-48
vs % Vermiculite (5% O_2)	+7	+15
High Turbulence Syst.		
vs % Vermiculite/Air	-20	-45
vs % Slow Current Aq/Air	+11	+18
Clinostat 2		
vs % Vertical Static	-74	-20
vs % Horizontal Static	-67	-35

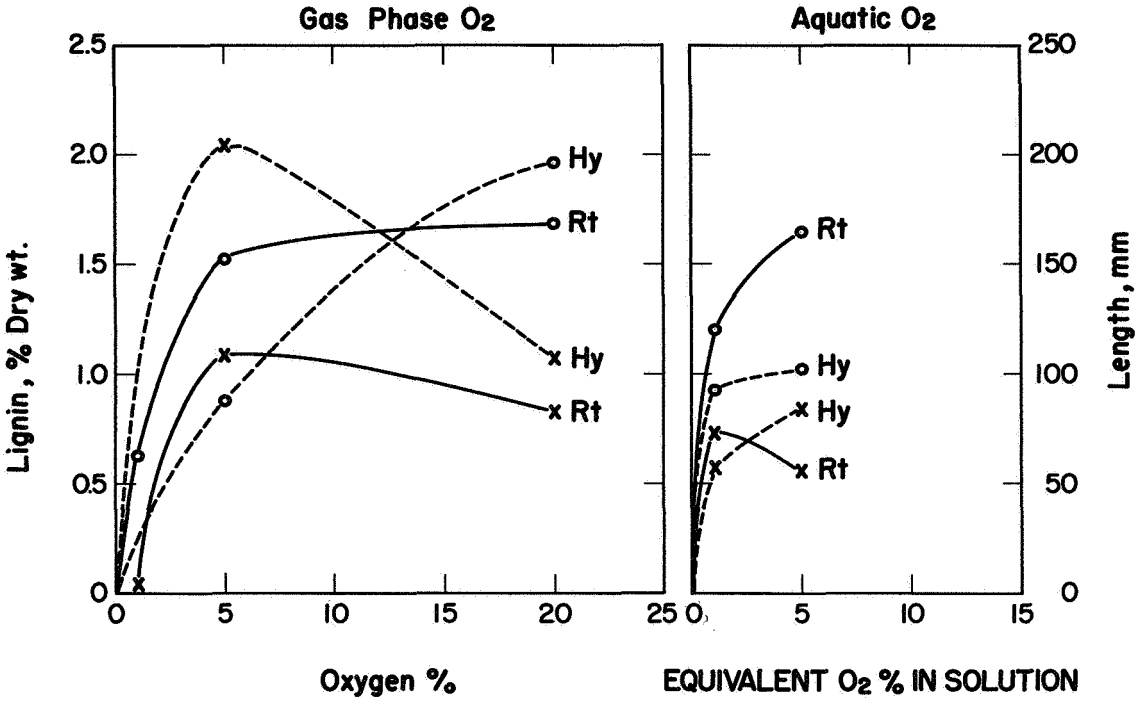

Fig. B-2-7. Oxygen relations in lignin content and linear growth in 7-day cucumber seedlings in gas phase and aquatic cultures. ○ lignin ✕ growth